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(54) Title: COMPOSITIONS FOR THE DETECTION OF ENZYME ACTIVITY IN BIOLOGICAL SAMPLES AND METHODS OF USE THEREOF

(57) Abstract

The present invention provides for novel reagents whose fluorescence increases in the presence of particular proteases. The reagents comprise a characteristically folded peptide backbone each end of which is conjugated to a fluorophore. When the folded peptide is cleaved, as by digestion with a protease, the fluorophores provide a high intensity fluorescent signal at a visible wavelength. Because of their high fluorescence signal in the visible wavelengths, these protease indicators are particularly well suited for detection of protease activity in biological samples, in particular in frozen tissue sections. Thus this invention also provides for methods of detecting protease activity *in situ* in frozen sections.

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COMPOSITIONS FOR THE DETECTION OF ENZYME ACTIVITY IN BIOLOGICAL SAMPLES AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of USSN 08/802,981, filed on February 20, 1997 which is herein incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

This invention pertains to a class of novel fluorogenic compositions whose fluorescence level increases in the presence active proteases. These fluorogenic protease indicators typically fluoresce at visible wavelengths and are thus highly useful for the detection and localization of protease activity in biological samples.

BACKGROUND OF THE INVENTION

Proteases represent a number of families of proteolytic enzymes that catalytically hydrolyze peptide bonds. Principal groups of proteases include metalloproteases, serine porteases, cysteine proteases and aspartic proteases. Proteases, in particular serine proteases, are involved in a number of physiological processes such as blood coagulation, fertilization, inflammation, hormone production, the immune response and fibrinolysis.

Numerous disease states are caused by and can be characterized by alterations in the activity of specific proteases and their inhibitors. For example emphysema, arthritis, thrombosis, cancer metastasis and some forms of hemophilia result from the lack of regulation of serine protease activities (*see, for example, Textbook of Biochemistry with Clinical Correlations*, John Wiley and Sons, Inc. N.Y. (1993)). In case of viral infection, the presence of viral proteases have been identified in infected cells. Such viral proteases include, for example, HIV protease associated with AIDS and NS3 protease associated with Hepatitis C. These viral proteases play a critical role in the virus life cycle.

Proteases have also been implicated in cancer metastasis. Increased synthesis of the protease urokinase has been correlated with an increased ability to

metastasize in many cancers. Urokinase activates plasmin from plasminogen which is ubiquitously located in the extracellular space and its activation can cause the degradation of the proteins in the extracellular matrix through which the metastasizing tumor cells invade. Plasmin can also activate the collagenases thus promoting the degradation of the collagen in the basement membrane surrounding the capillaries and lymph system thereby allowing tumor cells to invade into the target tissues (Dano, *et al.* (1985) *Adv. Cancer. Res.*, 44: 139.

Clearly measurement of changes in the activity of specific proteases is clinically significant in the treatment and management of the underlying disease states. Proteases, however, are not easy to assay. Typical approaches include ELISA using antibodies that bind the protease or RIA using various labeled substrates. With their natural substrates assays are difficult to perform and expensive. With currently available synthetic substrates the assays are expensive, insensitive and nonselective. In addition, many "indicator" substrates require high quantities of protease which results, in part, in the self destruction of the protease.

Recent approaches to protease detection rely on a cleavage-induced spectroscopic change in a departing chromogen or fluorogen located in the P1' position (the amino acid position on the carboxyl side of the cleavable peptide bond) (*see*, for example U.S. Patent Nos. 4,557,862 and 4,648,893). However, many proteases require two or four amino acid residues on either side of the scissile bond for recognition of the protease (a specific protease may require up to 6 amino acid residues) and thus, these approaches lack protease specificity.

Recently however, fluorogenic indicator compositions have been developed in which a "donor" fluorophore is joined to an "acceptor" chromophore by a short bridge containing a (7 amino acid) peptide that is the binding site for an HIV protease and linkers joining the fluorophore and chromophore to the peptide (Wang *et al.* (1990) *Tetra. Letts.* 45: 6493-6496). The signal of the donor fluorophore was quenched by the acceptor chromophore through a process believed to involve resonance energy transfer (RET). Cleavage of the peptide resulted in separation of the chromophore and fluorophore, removal of the quench and a subsequent signal was measured from the donor fluorophore.

Unfortunately, the design of the bridge between the donor and the acceptor led to relatively inefficient quenching limiting the sensitivity of the assay. In

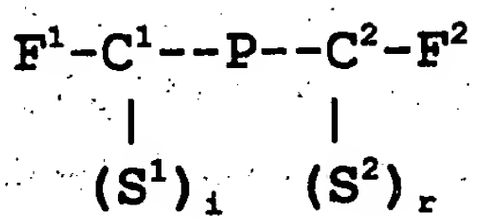
addition, the chromophore absorbed light in the ultraviolet range reducing the sensitivity for detection in biological samples which typically contain molecules that absorb strongly in the ultraviolet.

Clearly fluorogenic protease indicators that show a high signal level when cleaved, and a very low signal level when intact, that show a high degree of protease specificity, and that operate exclusively in the visible range thereby rendering them suitable for use in biological samples are desirable. The compositions of the present invention provide these and other benefits.

SUMMARY OF THE INVENTION

The present invention provides for novel reagents whose fluorescence increases in the presence of particular proteases. These fluorogenic protease indicators provide a high intensity fluorescent signal at a visible wavelength when they are digested by a protease. Because of their high fluorescence signal in the visible wavelengths, these protease indicators are particularly well suited for detection of protease activity in biological samples, in particular, in frozen tissue sections. The measurement can be carried out using a fluorescence microscope for histological samples and using a flow cytometer for cell suspension samples. Hence, the fluorogenic compositions of this invention allow detection of intracellular protease activity.

The fluorogenic protease indicators of the present invention are compositions suitable for detection of the activity of a protease. These compositions have the general formula:



I

in which P is a peptide comprising a protease binding site for said protease consisting of 2 to about 15, preferably 2 to about 12, preferably 2 to about 10, preferably 2 to about 8, 2 to about 6, or 2 to about 4 amino acids; F^1 and F^2 are fluorophores; S^1 and S^2 are peptide spacers ranging in length from 1 to about 50 amino acids; n and k are independently 0 or 1; and C^1 and C^2 are conformation determining regions comprising peptides ranging in length from 1 to about 8, amino acids, more preferably from 1 to about 6 amino acids. The conformation determining regions each introduce a bend

into the composition or otherwise restrict the degrees of freedom of the peptide backbone, thereby juxtaposing the fluorophores with a separation of less than about 100 Å. When either of the spacers (S^1 and S^2) are present they are linked to the protease binding site by a peptide bond to the alpha carbon of the terminal amino acid. Thus, when i is 1, S^1 is joined to C^1 by a peptide bond through a terminal α-amino group of C^1, and when r is 1, S^2 is joined to C^2 by a peptide bond through a terminal alpha carboxyl group of C^2.

The amino acid residues comprising a protease binding site are, by convention, numbered relative to the peptide bond hydrolyzed by a particular protease. Thus the first amino acid residue on the amino side of the cleaved peptide bond is designated P_1 while the first amino acid residue on the carboxyl side of the cleaved peptide bond is designated P_1'. The numbering of the residues increases with distance away from the hydrolyzed peptide bond. Thus a four amino acid protease binding region would contain amino acids designated:

$$P_2-P_1-P_1'-P_2'$$

and the protease would cleave the binding region between P_1 and P_1'.

In particularly preferred embodiments, the fluorogenic compositions of this invention are compositions of Formula II and Formula V as described herein. Preferred fluorophores have conformation determining regions and, optionally, spacers as described herein. In a most preferred embodiment, the compositions bear a single species of fluorophore. Fluorophores suitable for these "homolabeled" compositions include fluorophores that form H-type dimers. Particularly preferred fluorophores have an excitation wavelength between about 315 nm and about 700 nm.

In another embodiment, this invention provides methods of detecting the activity of a protease. The methods involve contacting the protease with one or more of the protease indicators described herein. In a particularly preferred embodiment, the "contacting" is in a histological section or in a cell suspension or culture derived from a biological sample selected from the group consisting of a tissue, blood, urine, saliva, or other biofluid, lymph, biopsy. The detection method can include a method selected from the group consisting of fluorescence microscopy, fluorescence microplate reader, flow cytometry, fluorometry, absorption spectroscopy.

In the compositions F^1 may be 5- and/or 6-carboxytetramethylrhodamine; and F^2 may be rhodamine X acetamide. These compositions may be conjugated to a solid support or to a lipid including membrane lipids or liposomes.

In another embodiment, any of the compositions described above may be used in a method for detecting protease activity in a sample. The sample may be a sample of "stock" protease, such as is used in research or industry, or it may be a biological sample. Thus, this invention provides for a method of detecting protease activity in a sample by contacting the sample with any of the compositions described above and then detecting a change in fluorescence of the fluorogenic composition where an increase in fluorescence indicates protease activity. The sample is preferably a biological sample which may include biological fluids such as sputum or blood, tissue samples such as biopsies or sections, and cell samples either as biopsies or in culture. Particularly preferred are tissue sections, cultured cells, cultured tissues, and the like.

This invention also provides a method of detecting a change in conformation of a molecule. The method involves: 1) providing a first molecule having attached thereto a first fluorophore and a second fluorophore wherein the first fluorophore and second fluorophore are the same species of fluorophore, and the fluorophores are juxtaposed at a distance sufficient for the interaction of said fluorophores to detectably reduce the fluorescence intensity of each of said fluorophores as compared to the fluorescence intensity of a single fluorophore attached to the molecule at the same location; and 2) detecting the change in fluorescence as the spacing between said fluorophores is increased by a change in conformation of the molecule. Alternatively the change in fluorescence as the relative orientation of the fluorophore is altered by a change in conformation of the molecule may be detected. Preferred fluorophores are capable of forming H-type dimers before the change in conformation. In a preferred embodiment the fluorophores are situated at a distance of less than about 10 angstroms from each other prior to the change in conformation. Particularly preferred fluorophores include the fluorophores described herein. In one embodiment, the change in conformation is cleavage of the molecule into two different molecules each bearing one of the fluorophores. In another embodiment the change in conformation is caused by binding of a target molecule to

said first molecule. In one embodiment, the first molecule is a nucleic acid and the change in conformation is produced by hybridization of the nucleic acid to a second nucleic acid or by binding of the nucleic acid to a transcription factor. In another embodiment, the first molecule is a polysaccharide and the change in conformation is produced by binding of an oligosaccharide binding molecule, *e.g.*, a lecithin binding protein. Preferred "backbone" molecules for this method include a nucleic acid, a polysaccharide, a peptide, a lipid, a protein, a phospholipid, a glycolipid, a glycoprotein, a steroid, or a polymer containing a pH or thiol-sensitive bond where the fluorophores attachment sites sandwich this bond. Where the backbone molecule is a nucleic acid, the change in conformation can be produced by hybridization of the nucleic acid to another nucleic acid or by cleavage of the nucleic acid (*e.g.*, by a restriction endonuclease or a ribozyme). Also the change in conformation can be produced by formation of a complex between the labeled oligonucleotide and a nucleotide binding protein. Where the backbone molecule is a peptide, polysaccharide or a lipid, the change in conformation can be produced by cleavage of the backbone molecule or by complex formation between the backbone molecule and its binding molecule such as an antibody, receptor, sugar binding protein, or lipid binding protein.

This invention also provides a fluorogenic composition for the detection of a change in conformation of said composition. In one embodiment, the fluorogenic composition comprising a molecule having attached thereto a first fluorophore and a second fluorophore wherein said first fluorophore and said second fluorophore are the same species of fluorophore, and said fluorophores are juxtaposed at a distance sufficient for the interaction of said fluorophores to detectably reduce the fluorescence intensity of each of said fluorophores as compared to the fluorescence intensity of each individual fluorophore attached to the molecule at the same location. The fluorophores are preferably fluorphores that form H-type dimers.

In still yet another embodiment, this invention provides a method of delivering a molecule into a cell. The method involves providing the molecule attached to at least two fluorophore molecules and a hydrophobic group; and contacting the cell with the molecule whereby th mol cule enters the cell. In one embodiment, the method involves providing the molecule attached to at least two largely flat hydrophobic fluorophore molecules and a hydrophobic group. Preferred

molecules include a polypeptide, a nucleic acid, a lipid, an oligosaccharide. Suitable fluorophores and hydrophobic groups ar described herein. Preferred cells include mammalian cells.

Definitions

The term "protease binding site" is used herein to refers to an amino acid sequence that is characteristically recognized and cleaved by a protease. The protease binding site contains a peptide bond that is hydrolyzed by the protease and the amino acid residues joined by this peptide bond are said to form the cleavage site. These amino acids are designated P_1 and P_1' for the residues on the amino and carboxyl sides of the hydrolyzed bond respectively.

A fluorophore is a molecule that absorbs light at a characteristic wavelength and then re-emits the light most typically at a characteristic different wavelength. Fluorophores are well known to those of skill in the art and include, but are not limited to rhodamine and rhodamine derivatives, fluorescein and fluorescein derivatives, coumarins and chelators with the lanthanide ion series. A fluorophore is distinguished from a chromophore which absorbs, but does not characteristically re-emit light.

"Peptides" and "polypeptides" are chains of amino acids whose α carbons are linked through peptide bonds formed by a condensation reaction between the α carboxyl group of one amino acid and the amino group of another amino acid. The terminal amino acid at one end of the chain (amino terminal) therefore has a free amino group, while the terminal amino acid at the other end of the chain (carboxy terminal) has a free carboxyl group. As used herein, the term "amino terminus" (abbreviated N-terminus) refers to the free α-amino group on an amino acid at the amino terminal of a peptide or to the α-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the peptide. Similarly, the term "carboxy terminus" refers to the free carboxyl group on the carboxy terminus of a peptide or the carboxyl group of an amino acid at any other location within the peptide. Peptides also include peptide mimetics such as amino acids joined by an ether as opposed to an amide bond.

The polypeptides described herein are written with the amino terminus at the left and the carboxyl terminus at the right. The amino acids comprising the

peptide compon nts of this inv ntion are numbered with resp ct to the protease cleavage site, with numbers increasing consecutively with distance in both the carboxyl and amino direction from the cleavage site. Residues on the carboxyl site are either notated with a "'" as in P_1', or with a letter and superscript indicating the region in which they are located. The "'" indicates that residues are located on the carboxyl side of the cleavage site.

The term "residue" or "amino acid" as used herein refers to an amino acid that is incorporated into a peptide. The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

The term "domain" or "region" refers to a characteristic region of a polypeptide. The domain may be characterized by a particular structural feature such as a ß turn, an alpha helix, or a ß pleated sheet, by characteristic constituent amino acids (*e.g.* predominantly hydrophobic or hydrophilic amino acids, or repeating amino acid sequences), or by its localization in a particular region of the folded three dimensional polypeptide. As used herein, a region or domain is composed of a series of contiguous amino acids.

The terms "protease activity" or "activity of a protease" refer to the cleavage of a peptide by a protease. Protease activity comprises the "digestion" of one or more peptides into a larger number of smaller peptide fragments. Protease activity of particular proteases may result in hydrolysis at particular peptide binding sites characteristically recognized by a particular protease. The particular protease may be characterized by the production of peptide fragments bearing particular terminal amino acid residues.

The amino acids referred to herein are described by shorthand designations as shown in Table 1.

Table 1. Amino acid nomenclature.

Name	Abbreviation	
	3 Letter	1 Letter
Alanine	Ala	A
βAlanine (NH_2-CH_2-CH_2-COOH)	βAla	
Arginine	Arg	R
Asparagine	Asn	N

Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Homoserine	Hse	-
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Methionine sulfoxide	Met (O)	-
Methionine methylsulfonium	Met (S-Me)	-
Norleucine	Nle	-
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
episilon-aminocaproic acid (NH^2-$(CH_2)_5$-COOH)	Ahx	J
4-aminobutanoic acid (NH_2-$(CH_2)_3$-COOH)	gAbu	
tetrahydroisoquinoline-3-carboxylic acid		O
Lys(N(epsilon)-trifluoroacetyl)		K[TFA]
α-aminoisobutyric acid	Aib	B

Other abbreviations used herein include "Fm" for Fmoc (9-fluorenylmethoxycarbonyl) group, "Ac" for N(alpha)-acetyl group, "daa" where "d" indicates the d isomer of the aa, and "Z" for benzyloxycarbonyl group.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, and 1C show an HPLC analysis of the D-NorFES-A protease indicator (F^1-Asp-Ala-Ile-Pro-Nle-Ser-Ile-Pro-Cys-F^2) where F^1 is a donor (D) fluorophore (5'-carboxytetramethylrhodamine (C2211) and F^2 is an acceptor (A) fluorophore (rhodamine X acetamide (R492))) before and after the addition of elastase. Fig. 1A: HPLC before the addition of elastase showing the late eluting peak representing the intact indicator molecule. Fig. 1B: HPLC after the addition of elastase with detection at 550 nm where both fluorophores absorb. Fig. 1C HPLC after the addition of elastase with detection at 580 nm where F^2 absorbs maximally.

Figures 2A and 2B show the emission spectra of the D-NorFES-A fluorogenic protease indicator (Fig. 2A) before and (Fig. 2B) after the addition of elastase.

Figure 3 shows the time-dependent increase of the fluorogenic protease indicator of Figure 1, as a function of time after addition of 1 unit of elastase.

Figures 4A and 4B show the fluorescence intensity of the donor fluorophore as a function of time after addition of 1 unit of elastase. Fig. 4A: The fluorogenic protease indicator of Figure 1. Fig. 4B: The peptide backbone of the fluorogenic protease of Figure 1 singly labeled with each of the two fluorophores. D-NorFES-A is the F^1-Asp-Ala-Ile-Pro-Nle-Ser-Ile-Pro-Cys-F^2 protease indicator where F^1 is a donor fluorophore (5'-carboxytetramethylrhodamine (C2211) and F^2 is an acceptor fluorophore (rhodamine X acetamide (R492). D-NorFES and A-NorFES each designate a molecule having the same peptide backbone, but bearing only one of the two fluorophores.

Figure 5 illustrates fluorescence of a DEVD, a DEVN, and an ICE substrate. To one hundred µl of assay buffer (50mM HEPES buffer pH 7.5, 10% (w/v) sucrose and 0.1% (w/v) CHAPS) containing 1µM of substrate DEVD (compound 2 of Example 8), DEVN (compound 3 of Example 8) and ICE (compound 5 of Example 8) 10µl of Jurkat cell lysate was added and incubated for 16 hours at 37°C. The Jurkat cells' lysate was prepared from the cells that had been stimulated by antiFas antibody at 1µg/ml concentration for 6 hours. The fluorescence intensity for the substrate solution alone is indicated in Figure 5 as a horizontal lined bar marked as t=0 hr and the fluorescence intensity of the lysate and substrate solution mixture after 16 hr is indicated by vertical line bar and is marked as t=16 hr digestion. 10 µl cell

lysate was pre-incubated with 50 µm ZVAD-FMK (benzyoxycarbonyl valanyl alanyl aspartyl-fluoromethylketone) at 37 d gree C for 30 min. then added to the substrate solution. The fluorescence intensity after 16 hours for this mixtur is indicated by the bar marked as ZVAD-FMK (inhibitor). Lastly, pre-incubated cell lysate with iodoacetamide(alkylating agent for sulfhydryl group) and PMSF (for inhibiting serine proteases) was added to the substrate solution. The fluorescence intensity after 16 hours at 37°C is indicated by bar marked as Iodoacetamide/PMSF. The DEVN substrate is a negative control substrate where the P1, Asp, residue is replaced by Asn. The CPP32 protease requires the P1 residue to be aspartic acid residue. The four bar graphs for the DEVN substrate (Fig. 5) clearly indicate that the activated cell lysate do not contain any other protease that digest the DEVD substrate, since the intensity for 16 hour digestion is the same as the substrate alone. The bar graphs for the DEVD substrate indicate that the activate cell lysate do contain CPP32 protease and this protease activities are inhibited by ZVAD-FMK, known CPP32 protease inhibitor. The contribution of any other proteases in digesting DEVD substrate is very small as indicated by the difference between the intensities of ZVAD-FMK bar to Iodoacetamide/PMSF bar.

DETAILED DESCRIPTION

Fluorogenic Indicators of Protease Activity

This invention provides for novel fluorogenic molecules useful for detecting protease activity in a sample. The fluorogenic protease indicators of the present invention generally comprise a fluorophore (donor) linked to an "acceptor" molecule by a peptide having an amino acid sequence that is recognized and cleaved by a particular protease. The donor fluorophore typically is excited by incident radiation at a particular wavelength which it then re-emits at a different (longer) wavelength. When the donor fluorophore is held in close proximity to the acceptor molecule, the acceptor absorbs the light re-emitted by the fluorophore thereby quenching the fluorescence signal of the donor molecule. The quench occurs whether the two fluorophores are different or the same species. Thus, in addition to peptides double labeled with two different fluorophores as shown in Example 1, peptides double labeled with the same fluorophore may also be used as protease indicators (*see, e.g.*, Example 6). Cleavage of a well-designed (*i.e.* a peptide of this

invention) joining the donor fluorophore and the acceptor results in separation of the two molecules, release of the quenching effect and increase in fluorescence.

In one basic application, the fluorogenic molecules of this invention may be used to assay the activity of purified protease made up as a reagent (*e.g.* in a buffer solution) for experimental or industrial use. Like many other enzymes, proteases may loose activity over time, especially when they are stored as their active forms. In addition, many proteases exist naturally in an inactive precursor form (*e.g.* a zymogen) which itself must be activated by hydrolysis of a particular peptide bond to produce the active form of the enzyme prior to use. Because the degree of activation is variable and because proteases may loose activity over time, it is often desirable to verify that the protease is active and to often quantify the activity before using a particular protease in a particular application.

Previous approaches to verifying or quantifying protease activity involve combining an aliquot of the protease with its substrate, allowing a period of time for digestion to occur and then measuring the amount of digested protein, most typically by HPLC. This approach is time consuming, utilizes expensive reagents, requires a number of steps and entails a considerable amount of labor. In contrast, the fluorogenic reagents of the present invention allow rapid determination of protease activity in a matter of minutes in a single-step procedure. An aliquot of the protease to be tested is simply added to, or contacted with, the fluorogenic reagents of this invention and the subsequent change in fluorescence is monitored (*e.g.*, using a fluorimeter or a fluorescence microplate reader).

In addition to determining protease activity in "reagent" solutions, the fluorogenic compositions of the present invention may be utilized to detect protease activity in biological samples. The term "biological sample", as used herein, refers to a sample obtained from an organism or from components (*e.g.*, cells) of an organism. The sample may be of any biological tissue or fluid. Frequently the sample will be a "clinical sample" which is a sample derived from a patient. Such samples include, but are not limited to, sputum, blood, blood cells (*e.g.*, white cells), tissue or fine needle biopsy samples, urine, peritoneal fluid, and pleural fluid, or cells therefrom. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes.

Previously described fluorogenic protease indicators typically absorb light in the ultraviolet range (*e.g.*, Wang *et al., supra.*). They are thus unsuitable for sensitive detection of protease activity in biological samples which typically contain constituents (*e.g.*, proteins) that absorb in the ultraviolet range. In contrast, the fluorescent indicators of the present invention both absorb and emit in the visible range (400 nm to about 750 nm). These signals are therefore not readily quenched by, nor is activation of the fluorophores, that is, absorption of light, interfered with by background molecules; therefore they are easily detected in biological samples.

In addition, unlike previous fluorogenic protease indicators which often utilize a fluorophore and a quenching chromophore, the indicators of the present invention may utilize two fluorophores (*i.e.*, fluorophore as both donor and acceptor) or the same two fluorophores effectively forming a ground-state dimer when joined by the one of the peptide backbones of this invention. Pairs of fluorophores may be selected that show a much higher degree of quenching than previously described chromophore/fluorophore combinations. In fact, previous compositions have been limited to relatively low efficiency fluorophores because of the small degree of quenching obtainable with the matching chromophore (Wang *et al. supra.*). In contrast, the fluorogenic protease indicators of this invention utilize high efficiency fluorophores and are able to achieve a high degree of quenching while providing a strong signal when the quench is released by cleavage of the peptide substrate. The high signal allows detection of very low levels of protease activity. Thus the fluorogenic protease indicators of this invention are particularly well suited for *in situ* detection of protease activity.

The fluorogenic protease indicators of the present have the general formula:

$$F^1-C^1--P--C^2-F^2 \quad \text{I}$$
$$\quad\quad |\quad\quad\quad |$$
$$(S^1)_n \quad (S^2)_x$$

where P is a peptide comprising a protease binding site, F^1 and F^2 are fluorophores, C^1 and C^2 are conformation determining regions, and S^1 and S^2 are optional peptide spacers. F^1 may be the donor fluorophore while F^2 is the acceptor fluorophore, or conversely, F^2 may be the donor fluorophore while F^1 is the acceptor fluorophore, or F^1 and F^2 may be identical. The protease binding site provides an amino acid

sequence (a peptide) that is recognized and cleaved by the protease whose activity th indicator is designed to reveal. The protease binding site is typically a peptide ranging in length from 2 amino acids to about 12 amino acids, 2 to about 10, 2 to about 8, 2 to about 6 or 2 to about 4 amino acids in length.

The conformation determining region is an amino acid sequence that introduces a bend into the molecule or otherwise restricts the degrees of freedom of the peptide backbone. The combined effect of the two conformation determining regions is to juxtapose the fluorophores attached to the amino and carboxyl termini of C^1 and C^2 respectively. The fluorophores are thus preferably positioned adjacent to each other at a distance less than about 100 angstroms. The fluorophores (F^1 and F^2) are typically conjugated directly to the conformation determining regions, although they may be joined by linkers. The optional spacers (S^1 and S^2) when present, are used to link the composition to a solid support or to anchor the composition to a component of a biological sample (*e.g.*, to a cellular membrane).

The substantially conformation determining regions increases the protease specificity of the composition. The amino acid sequences comprising the conformation determining regions are typically less accessible to the enzyme due to steric hinderance with each other and with the attached fluorophores. Conversely, the protease binding site is relatively unobstructed by either the fluorophore or the conformational determining region and is thus readily accessible to the protease.

Protease Binding Site and Conformation Determining Regions

The protease binding site and conformation determining regions form a contiguous amino acid sequence (peptide). The protease binding site is an amino acid sequence that is recognized and cleaved by a particular protease. It is well known that various proteases cleave peptide bonds adjacent to particular amino acids. Thus, for example, trypsin cleaves peptide bonds following basic amino acids such as arginine and lysine and chymotrypsin cleaves peptide bonds following large hydrophobic amino acid residues such as tryptophan, phenylalanine, tyrosine and leucine. The serine protease elastase cleaves peptide bonds following small hydrophobic residues such as alanine.

A particular protease, however, will not cleave every bond in a protein that has the correct adjacent amino acid. Rather, th proteases are specific to

particular amino acid sequenc s which serve as recognition domains for each particular protease. Without being bound by a particular theory, it is believed that a specific protease's preference for a particular cleavage site over many other potential sites in a folded globular protein may be largely determined by the potential cleavage sites' amino acid sequences and also their conformation and conformational flexibility.

Thus, for example, one obtains limited proteolysis products, *e.g.*, ribonuclease-S (a noncovalent complex consisting of two polypeptide chains) from a single chain folded protein ribonuclease-A using a protease called subtilisin. Similarly, one obtains a two chain noncovalent complex, Staphylococal nuclease-T, from single chain Staphylococcal nuclease by trypsin digestion. Another example of a specific protease's preference for one substrate over others is the human fibroblast-type collagenase. This protease prefers type I over type III soluble collagen even though both substrates contain the same collagenase sensitive Gly-Ile or Gly-Leu bonds (*see, e.g.*, Birkedal-Hansen *et. al.* (1993) *Crit. Rev. in Oral Biology and Medicine* 4:197-250).

Any amino acid sequence that comprises a recognition domain and can thus be recognized and cleaved by a protease is suitable for the "protease binding site" of the fluorogenic protease indicator compositions of this invention. Known protease substrate sequences and peptide inhibitors of proteases posses amino acid sequences that are recognized by the specific protease they are cleaved by or that they inhibit. Thus known substrate and inhibitor sequences provide the basic sequences suitable for use in the protease recognition region. A number of protease substrates and inhibitor sequences suitable for use as protease binding domains in the compositions of this invention are indicated in Table 2. One of skill will appreciate that this is not a complete list and that other protease substrates or inhibitor sequences may be used.

The amino acid residues comprising the protease binding site are, by convention, numbered relative to the peptide bond hydrolyzed by a particular protease. Thus the first amino acid residue on the amino side of the cleaved peptide bond is designated P_1 while the first amino acid residue on the carboxyl side of the cleaved peptide bond is designated P_1'. The numbering of the residues increases

with distanc away from the hydrolyzed peptide bond. Thus a four amino acid protease binding region would contain amino acids designated:

$$P_2\text{-}P_1\text{-}P_1'\text{-}P_2'$$

and the protease would cleave the binding region between P_1 and P_1'.

In a preferred embodiment, the protease binding region of the fluorogenic protease indicators of the present invention is selected to be symmetric about the cleavage site. Thus, for example, where a binding region is

Ile-Pro-Met-Ser-ILe

(*e.g.* α-1 anti-trypsin) and the cleavage occurs between Met and Ser then a four amino acid residue binding region based on this sequence would be:

$$-\ P_2-\ P_1\ -\ P_1'\text{-}P_2'-$$
$$\text{-Pro-Met-Ser-ILe-}$$

Other examples of binding domains selected out of longer sequences are provided in Table 2. The remaining amino or carboxyl residues that are not within the protease binding domain may remain as part of the conformation determining regions subject to certain limitations as will be explained below. Thus, in the instant example, the amino terminal Ile may be incorporated into the C^1 conformation determining region.

Various amino acid substitutions may be made to the amino acids comprising the protease binding domain to increase binding specificity, to eliminate reactive side chains, or to reduce the conformational entropy (decrease degrees of freedom) of the molecule. Thus, for example, it is often desirable to substitute methionine (Met) residues, which bear a oxidizable sulfur, with norleucine. Thus, in the example given, a preferred protease binding region will have the sequence:

$$-\ P_2-\ P_1\ -\ P_1'\text{-}P_2'-$$
$$\text{-Pro-Nle-Ser-ILe-}$$

Conformation Determining Regions

Conformation determining regions (C^1 and C^2) are peptide regions on either end of the protease cleavage region that both stiffen and introduce bends into the peptide backbone of the fluorogenic protease indicator molecules of this invention. The combination of the two conformation determining regions and the relatively straight protease cleavage region produces a roughly U-shaped molecule with the cleavage site at the base (middle) of the "U". The t rm U-shaped is, of

course, approximate, the point being that, as described below, the fluorophores are held relatively rigidly in clos juxtaposition (*e.g.*, less than about 100 angstroms).

In one preferred embodiment, amino acids such as proline (Pro) and α-aminoisobutyric acid (Aib) are selected both to introduce bends into the peptide molecule and to increase the rigidity of the peptide backbone. The C^1 and C^2 domains are selected such that the "arms" of the U are rigid and the attached fluorophores are localized adjacent to each other at a separation of less than about 100 angstroms. In order to maintain the requisite stiffness of the peptide backbone and placement of the fluorophores, the conformation determining regions are preferably 4 amino acids in length or less, or alternatively are greater than about 18 amino acids in length and form a stable alpha helix conformation or a ß-pleated sheet.

A) Tetrapeptide binding site compositions.

In a preferred embodiment, the peptide backbone of the fluorogenic protease indicators of the present invention will comprise a tripeptide C^1 region, a tetrapeptide P region and a single amino acid or dipeptide C^2 region. These compounds may be represented by the formula:

$$F^1\text{-}C^1{}_5\text{-}C^1{}_4\text{-}C^1{}_3\text{-}P_2\text{-}P_1\text{-}P_1{}'\text{-}P_2{}'\text{-}Y \qquad \text{II}$$
$$\Big|$$
$$(S^1)_n$$

where Y is either

$$C^2{}_3\text{-}C^2{}_4\text{--}F^2 \qquad \text{III}$$
$$\Big|$$
$$(S^2)_k$$

or

$$C^2{}_3\text{--}F^2 \qquad \text{IV}$$
$$\Big|$$
$$(S^2)_k$$

In these formulas the peptide binding region is designated $\text{-}P_2\text{-}P_1\text{-}P_1{}'\text{-}P_2{}'\text{-}$, while the amino acid residues of conformation determining regions C^1 and C^2 are designated $\text{-}C^1{}_5\text{-}C^1{}_4\text{-}C^1{}_3\text{-}$ and $\text{-}C^2{}_3\text{-}C^2{}_4\text{-}$ respectively. The C^2 region may either be an amino acid or a dipeptide. Whether the C^2 region is a dipeptide or an amino acid, the F^2 fluorophore and the S^2 spacer, when present, are always coupled to the carboxyl terminal residue of C^2. When a spacer is present on the C^2 region, it is attached the carboxyl terminal residue of C^2 by a peptide bond to the α carboxyl group.

As indicated above, the conformation determining regions typically contain amino acid residues such as a proline (Pro) that introduce a bend into the molecule and increase its stiffness. One of skill in the art will appreciate, however that where the terminal residues of the protease binding region (P) are themselves bend-creating residues such as proline, it is not necessary to locate a bend-creating residue at the position closest to P in the C region attached to that terminus. The conformation determining regions are thus designed by first determining the protease binding region, as described above, determining the "left-over" residues that would lie in the conformation determining regions, and if necessary, modifying those residues according to the following guidelines:

1. If the P_2' site is not a Pro then C^2 is a dipeptide (Formula **III**) Pro-Cys, Aib-Cys, Pro-Lys, or Aib-Lys, while conversely, if the P_2' site is a Pro then C^2 is a single amino acid residue (Formula **IV**) Cys or Lys.
2. If the P_2 site is not a Pro then C^1 is a tripeptide consisting of Asp-C^1_4-Pro, Asp-C^1_4-Aib, Asp-Aib-Pro, Asp-Pro-C^1_3, Asp-Aib-C^1_3, Asp-Pro-Aib, or Asp-Aib-Aib, while if the P_2 site is a Pro residue then group C^1 is a tripeptide consisting of Asp-C^1_4-C^1_3 or Asp-C^1_4-Aib.
3. If the P_3 (C^1_3) residue is a Pro then C^1 is a tripeptide consisting of Asp-C^1_4-Pro or Asp-Aib-Pro.
4. If the P_4 (C^1_4) residue is a Pro then C^1 is a tripeptide consisting of Asp-Pro-C^1_3 or Asp-Pro-Aib.
5. If P_2 and C^1_3 are both not prolines then C^1 is a tripeptide consisting of Asp-Pro-C^1_3, Asp-Aib-C^1_3, Asp-C^1_4-Pro, Asp-C^1_4-Aib, Asp-Pro-Aib, or Asp-Aib-Pro.

As indicated above, any methionine (Met) may be replaced with a norleucine (Nle). A number of suitable peptide backbones consisting of C^1, P and C^2 are provided in Table 2.

Tabl 2. Illustration of the design of the conformation determining regions and protease binding sit based on known protease substrate and inhibitor sequences. Italics indicate residues that are added to create a bend and to increase rigidity of the conformation determining regions. Normal font indicates residues of the substrate or inhibitor that forms the protease binding site. The thick line indicates the location at which the protease binding site is cleaved.

Substrate/ Inhibitor	CDR (C^1)			Protease Binding Site (P)				CDR (C^2)	
	C^1_5	C^1_4	C^1_3	P_2	P_1	P_1'	P_2'	C^2_3	C^2_4
α-1 anti-trypsin	*Asp*	*Ala*	Ile	Pro	Met *Nle*	Ser	Ile	*Pro Aib*	Cys Lys
plasminogen activator inhibitor 2	*Asp*	Met *Aib* Pro	Thr *Aib Pro*	Gly	Arg	Thr	Gly	*Pro Aib*	Cys Lys
neutrophil leukocyte elastase inhibitor	*Asp*	Ala *Aib*	Thr *Aib Pro*	Phe	Cys	Met *Nle*	Leu	*Pro Aib*	Cys Lys
anti-plasmin inhibitor	*Asp*	*Aib*	Ser *Aib Pro*	Arg	Met *Nle*	Ser	Leu	*Pro Aib*	Cys Lys
anti α-1 thrombin	*Asp*	Ile *Aib*	Ala *Aib Pro*	Gly	Arg	Ser	Leu	*Pro Aib*	Cys Lys
α-1 antichymotrypsin	*Asp*	*Aib*	Thr *Aib Pro*	Leu	Leu	Ser	Leu	*Pro Aib*	Cys Lys
interstitial type III (human liver) collagen	*Asp*	Gly *Aib*	Pro *Aib*	Leu	Gly	Ile	Ala	*Pro Aib*	Cys Lys
type I collagen for collagenase Bovine α 1	Asp	Gly *Aib Pro*	Pro *Aib*	Gln	Gly	Ile	Leu	*Pro Aib*	Cys Lys
type I collagen chick α2	Asp	Gly *Aib Pro*	Pro *Aib*	Gln	Gly	Leu	Leu	*Pro Aib*	Cys Lys
human α1 type II collagen	Asp	Gly *Aib Pro*	Pro *Aib*	Gln	Gly	Ile	Ala	*Pro Aib*	Cys Lys
type III collagen - AIA	Asp	Gly *Aib Pro*	Pro *Aib*	Gln	Ala	Ile	Ala	*Pro Aib*	Cys Lys
type III collagen (human skin)	*Asp*	Gly *Aib Pro*	Pro *Aib*	Gln	Gly	Ile	Ala	*Pro Aib*	Cys Lys
human α 2 macroglobulin	*Asp*	Gly *Aib Pro*	Pro *Aib*	Glu	Gly	Leu	Arg	*Pro Aib*	Cys Lys

stromelysin cleavage sites of stromelysin-1d	*Asp*	Asp *Aib* *Pro*	Val *Aib* *Pro*	Gly	His	Phe	Arg	*Pro* *Aib*	Cys Lys
stromelysin cleavage sites of stromelysin-1	*Asp*	Asp *Aib* *Pro*	Thr *Aib* *Pro*	Leu	Glu	Val	Met *Nle*	*Pro* *Aib*	Cys Lys
stromelysin cleavage site of proteoglycan link protein	*Asp*	Arg *Aib* *Pro*	Ala *Aib* *Pro*	Ile	His	Ile	Gln	*Pro* *Aib*	Cys Lys
gelatinase type IV collagenase site of 72 K gelatinases	*Asp*	Asp *Aib* *Pro*	Val *Aib* *Pro*	Ala	Asn	Tyr	Asn	*Pro* *Aib*	Cys Lys
gelatinase type IV cleavage of gelatin	*Asp*	Gly *Aib* *Pro*	Pro *Aib*	Ala	Gly	Glu	Arg	*Pro* *Aib*	Cys Lys
gelatinase type IV cleavage of gelatin	*Asp*	Gly *Aib* *Pro*	Pro *Aib*	Ala	Gly	Phe	Ala	*Pro* *Aib*	Cys Lys
type III collagen (human skin)	*Asp*	Gly *Aib* *Pro*	Pro *Aib*	Gln	Gly	Leu	Ala	*Pro* *Aib*	Cys Lys
Human FIB-CL propeptide	*Asp*	Asp *Aib* *Pro*	Val *Aib* *Pro*	Ala	Gln	Phe	Val	*Pro* *Aib*	Cys Lys
Cathepsin D (Thyroglobulin Fragment Tg1)	Asp	Asp Aib Pro	Gly Pro Aib	His	Phe	Leu	Arg	*Pro* *Aib*	Cys Lys
Cathepsin D (Thyroglobulin Fragment Tg2)	Asp	Thr Aib Pro	Thr Pro Aib	Glu	Leu	Phe	Ser	*Pro* *Aib*	Cys Lys
Cathepsin D (Thyroglobulin Fragment Tg3)	Asp	Lys Aib Pro	Phe Pro Aib	leu	Ala	Phe	Leu	*Pro* *Aib*	Cys Lys
Cathepsin D (Thyroglobulin Fragment Tg4)	Asp	Phe Aib Pro	Ser Pro Aib	His	Phe	Val	Arg	*Pro* *Aib*	Cys Lys
Prostate Specific Antigen (PSA) (Seminolgelin, Sg) Sg1	Asp	Gln Aib Pro	Gln Pro Aib	Leu	Leu	His	Asn	*Pro* *Aib*	Cys Lys
Prostate Specific Antigen (PSA) (Seminolgelin, Sg) Sg2	Asp	Ser Aib Pro	Ile Pro Aib	Gln	Tyr	Thr	Tyr	*Pro* *Aib*	Cys Lys

Prostate Specific Antigen (PSA) (Seminolgelin, Sg) Sg3	Asp	Ser Aib Pro	Ser Pro Aib	Gln	Tyr	Ser	Asn	Pro Aib	Cys Lys
Prostate Specific Antigen (PSA) (Seminolgelin, Sg) Sg4	Asp	Ser Aib Pro	Ser Pro Aib	Ile	Tyr	Ser	Gln	Pro Aib	Cys Lys
Gelatin α1 (type 1)	Asp	Gly Aib Pro	Pro Aib	Ala	Gly	Val	Gln	Pro Aib	Cys Lys

[1] In a preferred embodiment, the sequence may be followed by an S_2 spacer of Gly-Tyr. Thus, for example, where C^2_4 is Lys, C^2_4-S_2 is Lys-Gly-Tyr.

B) Indicators having other binding sites.

In another preferred embodiment, the binding site (P) ranges from 2 to about 12 amino acids in length. It was a discovery of this invention, that somewhat larger conformation determining regions can sufficiently restrict the degrees of freedom of the indicator molecule, that the fluorophores are suitably quenched regardless of the amino acid sequence of the binding (recognition) domain (P). In one preferred embodiment, these compositions are include the compounds represented by the Formula V:

$$\underset{(S^1)_i}{\underset{|}{F_1}}\text{-}aa^1_j\text{-}(aa^2\text{-}aa^3)_k\text{-}aa^4_l\text{-}aa^5\text{-}X_m\text{-}P\text{-}Y_n\text{-}aa^6\text{-}aa^7_o\text{-}(aa^8\text{-}aa^9)\text{-}\underset{(S^2)_r}{\underset{|}{aa^{10}_q}}\text{-}F_2 \qquad \text{V}$$

In this formula, P is a peptide comprising a protease binding site and consists of 2 to about 12 amino acids, F^1 and F^2 are fluorophores where F^1 is attached to the amino terminal amino acid and F^2 is attached to the carboxyl terminal amino acid of the composition (excluding spacers). S^1 and S^2, when present, are peptide spacers ranging in length from 1 to about 50 amino acids and S^1, when present, is attached to the amino terminal amino acid, while S^2, when present, is attached to the carboxyl terminal amino acid. The subscripts i, j, k, l, m, n, o, p, q, and r are independently 0 or 1.

In a particularly preferred embodiment, aa^1 and aa^{10} are independently selected from the group consisting of lysine, ornithine and cysteine; aa^2, aa^3, aa^8 and aa^9 are independently selected from the group consisting of an amino acid or a dipeptid consisting of Asp, Glu, Lys, Ornithine, Arg, Citulline,

homocitrulline, Ser, homoserine, Thr, and Tyr; aa^5, aa^4, aa^6, and aa^7 are independently selected from the group consisting of proline, 3,4-dehydroproline, hydroxyproline, alpha aminoisobutyric acid and N-methyl alanine; X is selected from the group consisting of Gly, βAla, γAbu ,Gly-Gly, Ahx, βAla- Gly, βAla-βAla, γAbu-Gly, βAla-γAbu, Gly-Gly-Gly, γAbu-γAbu, Ahx-Gly, βAla-Gly-Gly, Ahx-βAla, βAla-βAla-Gly, Gly-Gly-Gly-Gly, Ahx-γAbu, βAla-βAla-βAla, γAbu-βAla-Gly, γAbu-γAbu-Gly, Ahx-Ahx, γAbu-γAbu-βAla, and Ahx-Ahx-Gly; Y is selected from the group consisting of Gly, βAla, γAbu, Gly-Gly, Ahx, Gly-βAla, βAla-βAla, Gly-γAbu, γAbu-βAla, Gly-Gly-Gly, γAbu-γAbu, Gly-Ahx, Gly-Gly-βAla,βAla-Ahx, Gly-βAla-βAla, Gly-Gly-Gly-Gly, γAbu-Ahx, βAla-βAla-βAla, Gly-βAla-γAbu, Gly-γAbu-γAbu, Ahx-Ahx, βAla-γAbu-γAbu, and Gly-Ahx-Ahx.

When i is 1, S^1 is joined to aa^1 by a peptide bond through a terminal alpha amino group of aa^1; and when r is 1, S^2 is joined to aa^{10} by a peptide bond through a terminal alpha carboxyl group of aa^{10}. It will be appreciated that amino acids 1-4 or 7-10 may be absent. When one or more of these amino acids are absent, the fluorophores are attached to the remaining terminal amino acids.

The amino acid backbones of such particularly preferred compositions are listed in Tables 3 and 4.

Table 3. Illustration of the design of the conformation determining regions and protease binding sites in molecules having P domains larger that 4 amino acids. The P1 residue is underlined. Z is benzyloxycarbonyl group, K[TFA] means Lys(N(epsilon)trifluoroacetyl), Fm is Fmoc (preferably attached to the alpha amino group of the amino terminal residue *e.,g.*, Lysine (K). O indicates tetrahydroisoquinoline-3-carboxylic acid. Aib, designated as B, can be replaced by Pro.

Name	aa^1	aa^2-aa^3	aa^4	aa^5	X	P	Y	aa^6	aa^7	aa^8-aa^9	aa^{10}	S^2
PAI-2	K	D		B		TGRTG		P			K	GY
PAI-2(b)	K	D	P	P		TGRTG		P	P		K	GY
DEVD	K	D		B		DEVDGID		P			K	GY
DevN	K	D		B		DEVNGID		P			K	GY
PARP	K	D		B		EVDGID		P			K	GY
ICE	K	DY		B		ADGID		P			K	GY
Fm-DEVD	Fm-K	D		B		DEVDGID		P			K	GY
Fm-DEVN	Fm-K	D		B		DEVNGID		P			K	GY
Fm-PARP	Fm-K	D		B		EVDGID		P			K	GY
Fm-KNFES	Fm-K	D		-		AIPMSI		P			K	GY
	Fm-K	D				AIPNIuSI		P			K	GY
Fm-G2D2D	Fm-K	D		B		GDEVDGID	G	P			K	GY
Fm-CGD2D	Fm-K	D		B	J	GDEVDGID	GJ	P			K	GY
Z-CGD2D	Z-K	D		B	J	GDEVDGID	GJ	P			K	GY
Fm-ICE	Fm-K	DY		B		ADGID		P			K	GY

Table 4. Illustration of the design of the conformation determining regions and protease binding sites in molecules having P domains larger that 4 amino acids. The P1 residue is underlined. Z is benzyloxycarbonyl group, K[TFA] means

Lys(N(epsilon)trifluoroacetyl), Fm is Fmoc (preferably attached to the alpha amino group of the amino terminal residue *e.,g.*, Lysine (K). O indicates tetrahydroisoquinoline-3-carboxylic acid. Aib, designated as B, can be replaced by Pro. J is a C

Substrate class	aa^1	aa^2-aa^3	aa^4	aa^5	X	P	Y	aa^6	aa^7	aa^8-aa^9	aa^{10}	S^2
CPP32 substrates (preferably with DER and TMR fluorophores). Note where Fmoc (Fm) is indicated, it is optional, and where not indicated it can be added.												
	Fm-K	DP			JG	DEVDGIN	GJ	P			K	GY
	Fm-K	DB			JG	DEVNGIN	GJ	P			K	GY
	Fm-K	DB			JG	DEVDGID	GJ	P			K	GY
	Fm-K	DB			JG	DEVDGIN	GJ	P			K	GY
	Fm-K	DB			JG	DEVNGID	GJ	P			K	GY
	K	D		B	JJ	GDEVDGID	JJ	P			K	GY
	K	D		B	J	GNEVDGID	GJ	P			K	GY
	K	D		B	J	GDEVDGIN	GJ	P			K	GY
	K	D		B	J	GNEVDGIN	GJ	P			K	GY
	K	D		B	J	GDEVNGIN	GJ	P			K	GY
	K	D		B	J	GNEVNGIN	GJ	P			K	GY
	K	D		B	JG	ODEVDGID	GJ	P			K	GK
	K	D		B	JG	dODEVDGID	GJ	P			K	GY
	K	D		B	JG	WDEVDGID	GJ	P			K	GY
	K	D		B	JG	dWDEVDGID	GJ	P			K	GY

	K	D		B	JG	dOdODEVDGID	GJ	P			K	GY
	K	D		B	JG	dWdWDEVDGID	GJ	P			K	GY
	K	D		B		YVADGID		P			K	GY
	K	D		B		YVADGIN		P			K	GY
	K	D		B		YVANGIN		P			K	GY
	K	D		B	G	YVADGID	G	P			K	GY
	K	D		B	G	YVADGIN	G	P			K	GY
	K	D		B	G	YVANGIN	G	P			K	GY
	K	D		B	JG	YVADGID	GJ	P			K	GY
	K	D		B	JG	YVANGID	GJ	P			K	GY
	K	D		B	JG	YVANGIN	GJ	P			K	GY
	K	D		B	JG	YVADGIN	GJ	P			K	GY
	K	D		B	JG	dYVADGIN	GJ	P			K	GY
LAMIN-A												
	K	D		B		LVEIDNG		P			K	GY
	K	D		B	G	LVEIDNG	G	P			K	GY
	K	D		B	JG	LVEIDNG	GJ	P			K	GY
	K	D		B	JG	LVEINNG	GJ	P			K	GY
ProCPP32Asp175												
	K	D		B		GIETDSGVDD		P			K	GY

	K	D		B		GIETNSGVDD		P			K	GY
	K	D		B	G	GIETDSGVDD	G	P			K	GY
	K	D		B	G	GIETNSGV	G	P			K	GY
	K	D		B	J	GIETDSGV	J	P			K	GY
	K	D		B	J	GIETNSGV	J	P			K	GY
	K	D		B	JG	GIETDSGV	GJ	P			K	GY
	K	D		B	JG	GIETNSGV	GJ	P			K	GY
ProCPP32Asp28												
	K	D		B		GSESMDSGISLD		P			K	GY
	K	D		B	G	GSESMDSG	G	P			K	GY
	K	D		B	JG	GSESMDSG	GJ	P			K	GY
NS3 NS5A/5B												
	K	D		B	JG	DVVCCSMS	GJ	P			K	GY
	K	D		B	JG	DVVCDSMS	GJ	P			K	GY
	K	D		B	JG	DVVCCSdMS	GJ	P			K	GY
	K	D		B	JG	DVVCDSdMS	GJ	P			K	GY
	K	D		B	JG	DVVCCPdMS	GJ	P			K	GY
	K	D		B	JG	EDVVCCS	GJ	P			K	GY
	K	D		B	JG	EDVVCDS	GJ	P			K	GY
	K	D		B	JG	EDdVVCCP	GJ	P			K	GY

	K	D		B	JG	EDdVVCDP	GJ	P			K	GY
	K	D		B	JG	DdVVCCSdMS	GJ	P			K	GY
	K	D		B	JG	DVdVCDSdMS	GJ	P			K	GY
	K	D		B	JG	DdVVCCPdMS	GJ	P			K	GY
	K	D		B	JG	DVVCCSM	GJ	P			K	GY
	K	D		B	JG	DVVCDSM	GJ	P			K	GY
	K	D		B	JG	VCCSM	GJ	P			K	GY
	K	D		B	JG	VCDSM	GJ	P			K	GY
NS3 NS4A/4B												
	K	D		B	JG	DEMEECSQHL		P			K	GY
	K	D		B	JG	DEMEECPQHL		P			K	GY
	K	D		B	JG	DEMEEDSQHL		P			K	GY
	K	D		B	JG	EMEECSQHL		P			K	GY
	K	D		B	JG	EMEECPQHL		P			K	GY
	K	D		B	JG	EMEEDSQHL		P			K	GY
	K	D		B	JG	EMEECSQHL	G	P			K	GY
	K	D		B	JG	EMEECPQHL	G	P			K	GY
	K	D		B	JG	EMEEDSQHL	G	P			K	GY
	K	D		B	JG	EMEECSQHL	GJ	P			K	GY
	K	D		B	JG	EMEECPQHL	GJ	P			K	GY

	K	D		B	JG	EMEEDSQHL	GJ	P			K	GY
Ext. PAI-2												
	K	D		B	JG	VMTGRTG	J	P			K	GY
	K	D		B	JG	VdMTGRTG	J	P			K	GY
	K	D		B	JG	VMTGRTG	J	P			K	GY
	K	D		B	JG	VMTGRTG	J	P			K	GY
THROMB												
	K	D		B	JG	VMTGRG	J	P			K	GY
	K	D		B	JG	VMTGRG	GJ	P			K	GY
	K	D		B	JG	VdmTGRG	GJ	P			K	GY
UROK												
	K	D		B	JG	VMTGRVG	J	P			K	GY
	K	D		B	JG	VdMTGRVG	J	P			K	GY
F12A												
	K	D		B	JG	VMTGRAG	J	P			K	GY
	K	D		B	JG	VdMTGRAG	J	P			K	GY
Swedish KM/NL AMLOID												
	K	D		B	JG	SEVNLDAEF	GJ	P			K	DDY
	K	D		B	JG	SEVKLDAEF	GJ	P			K	DDY
NATIVE AMYLOID												

	K	D		B	JG	SEVKMDAEF	GJ	P			K	DDY
CATHESPSIN G												
	K	D		B	JG	SEVKMDDEF	GJ	P			K	DDY
	K	D		B	JG	SEVNLDDEF	GJ	P			K	DDY
APP[709-710]												
	K	D		B	JG	GVVIATVIVIT	GJ	P			K	DDY
APP[708-719]												
	K	D		B	JG	YGVVIATVIVIT	GJ	P			K	DDY
APP[711-716]												
	K	D		B	JG	VIATVI	GJ	P			K	DDY
APP[708-713]												
	K	D		B	JB	YGVVIA	GJ	P			K	DDY
PSA Sg1												
	K	D		B	JJ	QQLLHN	JJ	P			K	
	K	D		B	JG	QQLLHN	GJ	P			K	
	K	D		B	G	QQLLHN	G	P			K	
	K	D		B		QQLLHN		P			K	
PSA Sg2												
	K	D		B	JJ	SIQYTY	JJ	P			K	
	K	D		B	JG	SIQYTY	GJ	P			K	

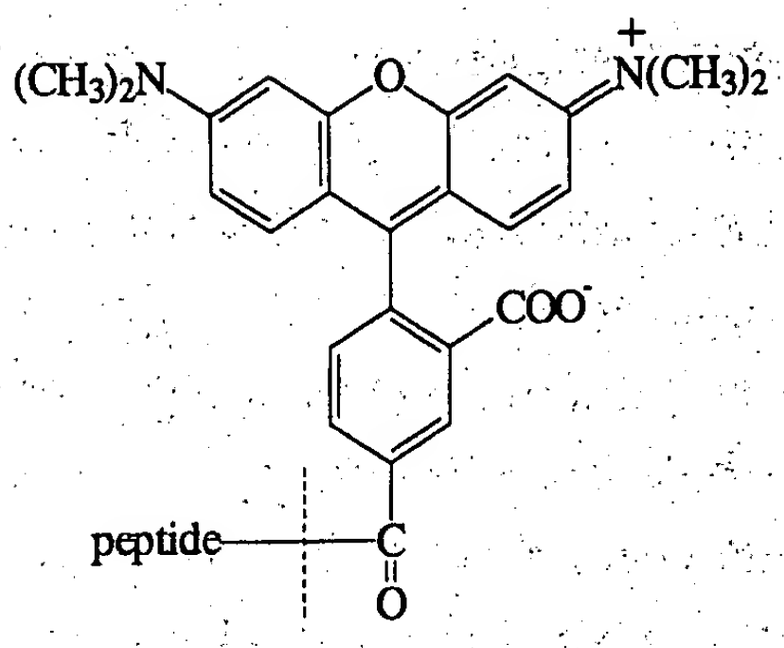
	K	D		B	G	SIQYTY	G	P			K	
	K	D		B		SIQYTY		P			K	
PSA Sg3												
	K	D		B	JJ	SSQYSN	JJ	P			K	
	K	D		B	JG	SSQYSN	GJ	P			K	
	K	D		B	G	SSQYSN	G	P			K	
	K	D		B		SSQYSN		P			K	
PSA Sg4												
	K	D		B	JJ	SSIYSQ	JJ	P			K	
	K	D		B	JG	SSIYSQ	GJ	P			K	
	K	D		B	G	SSIYSQ	G	P			K	
	K	D		B		SSIYSQ		P			K	
Cathepsin D substrates (preferably with diethylrhodamine fluorophore, note fmoc (Fm) is optional)												
	Fm-K	DP		-	JG	SEVNLDAEF	GJ	P			K	GY

"Donor" and "Accept r" Fluorophores

A fluorophore excited by incident radiation absorbs light and then subsequently re-emits that light at a different (longer) wavelength. However, in the presence of a second class of molecules, known as "acceptors" the light emitted by a so-called donor fluorophore is absorbed by the acceptor thereby quenching the fluorescence signal of the donor. Thus, use of two fluorophores, as opposed to a fluorophore/chomophore pair, allows a clearer assessment of the overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor. This facilitates the design of a peptide backbone that allows allowing optimization of the quenching. This results in a high efficiency donor/acceptor pair facilitating the detection of low concentrations of protease activity.. Thus, although a fluorophore/chromophore combination may be suitable, in a preferred embodiment, the fluorogenic protease inhibitors of this invention will comprise two fluorophores.

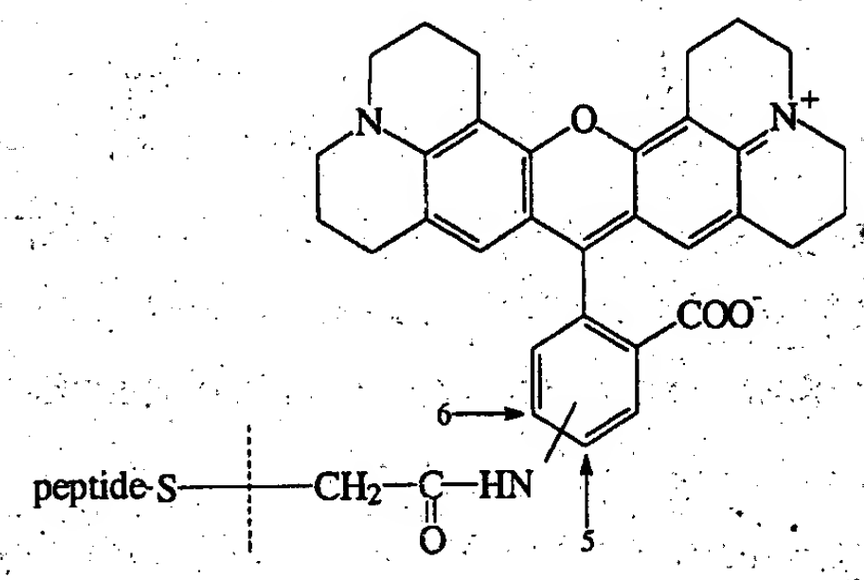
The "donor" and "acceptor" molecules are typically selected as a matched pair such that the absorption spectra of the acceptor molecule overlaps the emission spectrum of the donor molecule as much as possible. In addition, the donor and acceptor fluorophores are preferably selected such that both the absorption and the emission spectrum of the donor molecule is in the visible range (400 nm to about 700 nm). The fluorophores thereby provide a signal that is detectable in a biological sample thus facilitating the detection of protease activity in biological fluids, tissue homogenates, *in situ* in tissue sections, and the like. The emission spectra, absorption spectra and chemical composition of many fluorophores are well known to those of skill in the art (*see*, for example, *Handbook of Fluorescent Probes and Research Chemicals*, R.P. Haugland, ed. which is incorporated herein by reference).

Preferred fluorophore pairs include the rhodamine derivatives. Thus, for example 5-carboxytetramethylrhodamine or the succinimidyl ester of 5- and/or 6-carboxytetramethylrhodamine (9-(2,5-dicarboxyphenyl)-3,6-bis-(dimethylamino)xanthylium chloride (5-TMR) and 9-(2,6-dicarboxyphenyl)-3,6-bis-(dimethylamino)xanthylium chloride (6-TMR), C2211 and C1171 respectively, available from Molecular Probes, Eugene, Oregon, USA) (Formula VI) is a particularly preferred donor molecule



VI

and rhodamine X acetamide (R 492 from Molecular Probes) (Formula VII)



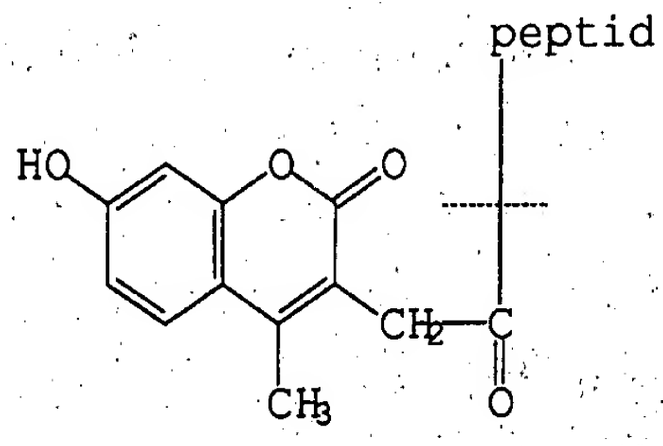
VII

or the succinimidyl ester of 5- and/or 6-carboxy-X-rhodamine (9-(2,5-dicarboxyphenyl)-2,7-dimethyl-3,6-bis(ethylamino)xanthene (5-DER) and 9-(2,6-dicarboxyphenyl)-2,7-dimethyl-3,6-bis(ethylamino)xanthene (6-DER), mixed isomer available as C1309 (designated herein as DER) from Molecular Probes) is a particularly preferred receptor molecule. These fluorophores are particularly preferred since the excitation and emission of both members of this donor/acceptor pair are in the visible wavelengths, the molecules have high extinction coefficients and the molecules have high fluorescence yields in solution. The extinction coefficient is a measure of the light absorbance at a particular wavelength by the

chromophore and is therefore related to its ability to quench a signal, while the fluorescence yield is the ratio of light absorbed to light re-emitted and is a measure of the efficiency of the fluorophore and thus effects the s nsitivity of the prot ase indicator.

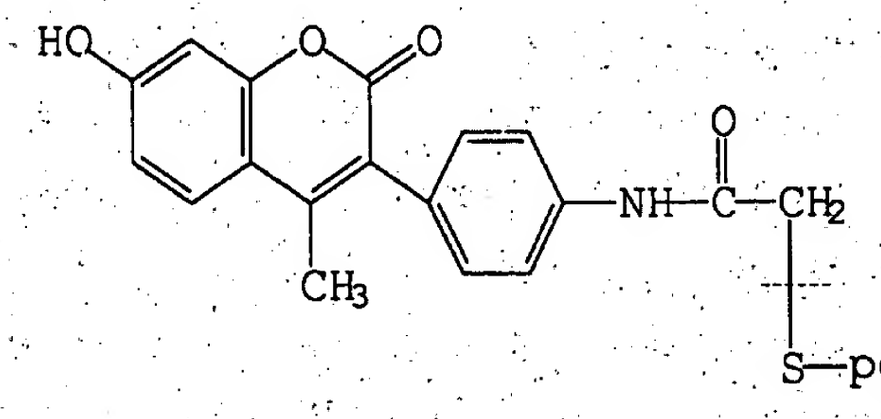
Other preferred fluorophores include, but are not limited to, 9-(2-carboxyphenyl)-2,7-dimethyl-3,6-bis(ethylamino)xanthylium, 9-(2-carboxyphenyl)-3,6-bis(dimethylamino)xanthylium, and 9-(2-carboxyphenyl)-xanthylium.

Of course, while not most preferred, fluorophores that absorb and emit in the ultraviolet may also be used in the protease indicators of the present invention. One particularly preferred ultraviolet absorbing pair of fluorophores is 7-hydroxy-4-methylcoumarin-3-acetic acid as the donor molecule (Formula **VIII**)



VIII

and 7-diethylamino-3-((4'-iodoacetyl)amino)phenyl)-4-methylcoumarin (Formula **IX**) as the acceptor molecule.



IX

These and other fluorophores are commercially available from a large number of manufacturers such as Molecular Probes (Eugene, Oregon, USA).

It was a surprising discovery that fluorophores having matched absorption and emission spectra are not required in the practice of the present invention. In fact, a single species of fluorophore, when joined to the polypeptide backbones of this invention in the positions occupied by F^1 and F^2, is capable of quenching itself. Moreover, this quenching is fully released when the peptide backbone is cleaved.

Without being bound to a particular theory, it is believed that quenching is accomplished by the formation of ground state dimers wherein the electron orbitals of the two fluorophores interact resulting in reciprocal quenching. It is the limited conformational entropy of the peptide backbones of this invention that forces fluorophores into close enough proximity to effectively form a ground state dimer.

Particularly preferred molecules form H-type dimers. The formation of H-type dimers by fluorescent molecules is described by Packard *et al.* (1996) *Proc. Natl. Acad. Sci. USA*, 93: 11640-11645. The H-type dimer is characterized by exciton bands in the absorption spectra and fluorescence quenching (*see, e.g.*, Valdes-Aguilera *et al.* (1989) *Acc. Chem. Res.*, 22: 171-177 and Packard *et al.* (1996) *Proc. Natl. Acad. Sci. USA*, 93: 11640-11645).

Thus, in a preferred embodiment, the protease indicators of this invention include only a single species of fluorophore, more preferably a fluorophore capable of forming H-type dimers.

NorFes is an undecapeptide that contains a recognition sequence and cleavage site for the serine protease elastase. When NorFes was doubly labeled with a variety of fluorophores on opposite sites of the amino acid sequence, the fluorescence was quenched due to formation of intramolecular ground-state dimers. The spectral characteristics of these dimers were predictable by exciton theory.

The decrease in dimer/monomer ratios as the temperature was raised indicated an intermolecular attraction between the dye molecules. The free energy of activation of disruption of homodimers composed of tetramethylrhodamine was at least 1.7 kcal/mole and for those of diethylrhodamine was 2.4 kcal/mnole. Because of the intermolecular attraction of fluorophores that form exciton dimers the connecting amino acid sequences can deviate from the optimal sequ nces described herein. Thus, when exciton-forming fluorphores are used, amino acid

substitutions can be made in the "backbones" described herein and activity can still be maintained.

Particularly preferred exciton-forming fluorophores include carboxytetramethylrhodamine, carboxyrhodamine-X, diethylaminocoumarin, and carbocyanine dyes. In this embodiment, there is no need to match emission or absorption spectra since only a single fluorophore is used. Thus a wide variety of fluorophores can be used effectively. In addition, the use of a single fluorophore greatly simplifies synthesis chemistry.

The use of homo-doubly labeled indicators (indicators labeled with a single species of fluorophore) of this invention also permits detection of enzymatic activity by absorbance measurements in addition to fluorescence measurements. Since blue-shifted exciton bands (or blue-shifted absorption maxima) in absorption spectra denote H-dimer formation and fluorescence quenching is concomitant with the latter, measurement of absorption spectra may be sufficient as a diagnostic tool in the proper setting. When a doubly labeled protease indicator is cleaved by a specific protease, the H-type dimer is disrupted. The blue shifted absorption maximum associated with the H-type dimer is then lost. Hence, if one measures the intensity of absorption at this blue shifted exciton band then as the H-type dimer is disrupted the absorption intensity is expected to decreased whereas the absorption intensity at the monomer maximum peak wavelength is expected to increase.

Preparation of Fluorogenic Protease Indicators

The fluorogenic protease indicators of the present invention are preferably prepared by first synthesizing the peptide backbone, *i.e.* the protease cleavage site (P), the two conformation determining regions (C^1 and C^2), and the spacers (S^1 and S^2) if present. The fluorophores are then chemically conjugated to the peptide. The fluorophores are preferably conjugated directly to the peptide however, they may also be coupled to the peptide through a linker. Finally, where the fluorogenic protease indicator is to be bound to a solid support, it is then chemically conjugated to the solid support via the spacer (S^1 or S^2) either directly or through a linker.

Preparation of th peptid backb n

Solid phase peptide synthesis in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence is the preferred method for preparing the peptide backbone of the compounds of the present invention. Techniques for solid phase synthesis are described by Barany and Merrifield, *Solid-Phase Peptide Synthesis;* pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part a.*, Merrifield, *et al. J. Am. Chem. Soc. 85*, 2149-2156 (1963), and Gross and Meienhofer, eds. Academic press, N.Y., 1980 and Stewart *et al., Solid Phase Peptide Synthesis*, 2nd ed. Pierce Chem. Co., Rockford, Ill. (1984) which are incorporated herein by reference. Solid phase synthesis is most easily accomplished with commercially available peptide synthesizers utilizing FMOC or TBOC chemistry. The chemical synthesis of the peptide component of a fluorogenic protease indicator is described in detail in Examples 1 and 2.

In a particularly preferred embodiment, peptide synthesis is performed using Fmoc synthesis chemistry. The side chains of Asp, Ser, Thr and Tyr are preferably protected using t-Butyl and the side chain of Cys residue using S-trityl and S-t-butylthio, and Lys residues are preferably protected using t-Boc, Fmoc and 4-methyltrityl for lysine residues. Appropriately protected amino acid reagents are commercially available. The use of multiple profecting groups allows selective deblocking and coupling of a fluorophore to any particular desired side chain. Thus, for example, t-Boc deprotection is accomplished using TFA in dichloromethane, Fmoc deprotection is accomplished using 20% (v/v) piperidine in DMF or N-methylpyrolidone, and 4-methyltrityl deprotection is accomplished using 1 to 5% (v/v) TFA in water or 1% TFA and 5% triisopropylsilane in DCM, S-t-butylthio deprotection is accomplished in aqueous mercaptoethanol (10%), t-butyl and t-boc and S-trityl deprotection is accomplished using TFA : phenol : water : thioanisol : ethanedithiol (85:5:5:2.5:2.5), and t-butyl and t-Boc deprotection is accomplished using TFA : phenol : water (95: 5: 5). Detailed synthesis, deprotection and fluorophore coupling protocols are provided in Examples 1 and 2.

Alternatively, the peptide components of the fluorogenic protease indicators of the present invention may be synthesized utilizing recombinant DNA

technology. Briefly, a DNA molecule encoding the desired amino acid sequence is synthesized chemically by a variety of methods known to those of skill in the art including the solid phase phosphoramidite method described by Beaucage and Carruthers, *Tetra. Letts.* 22: 1859-1862 (1981), the triester method according to Matteucci, *et al., J. Am. Chem. Soc.*, 103:3185 (1981), both incorporated herein by reference, or by other methods known to those of skill in the art. It is preferred that the DNA be synthesized using standard ß-cyanoethyl phosphoramidites on a commercially available DNA synthesizer using standard protocols.

The oligonucleotides may be purified, if necessary, by techniques well known to those of skill in the art. Typical purification methods include, but are not limited to gel electrophoresis, anion exchange chromatography (e.g. Mono-Q column, Pharmacia-LKB, Piscataway, New Jersey, USA), or reverse phase high performance liquid chromatography (HPLC). Method of protein and peptide purification are well known to those of skill in the art. For a review of standard techniques see, *Methods in Enzymology Volume 182: Guide to Protein Purification*, M. Deutscher, ed. (1990), pages 619-626, which are incorporated herein by reference.

The oligonucleotides may be converted into double stranded DNA either by annealing with a complementary oligonucleotide or by polymerization with a DNA polymerase. The DNA may then be inserted into a vector under the control of a promoter and used to transform a host cell so that the cell expresses the encoded peptide sequence. Methods of cloning and expression of peptides are well known to those of skill in the art. See, for example, Sambrook, *et al.*, *Molecular Cloning: a Laboratory Manual* (2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory (1989)), *Methods in Enzymology*, Vol. 152: *Guide to Molecular Cloning Techniques* (Berger and Kimmel (eds.), San Diego: Academic Press, Inc. (1987)), or *Current Protocols in Molecular Biology*, (Ausubel, *et al.* (eds.), Greene Publishing and Wiley-Interscience, New York (1987), which are incorporated herein by reference.

Linkage of the fluorophores to the peptide backbone

The fluorophores are linked to the peptide backbone by any of a number of means well known to those of skill in the art. In a preferred embodiment, the fluorophore is linked directly from a reactive site on the fluorophore to a reactive

group on th peptide such as a terminal amino or carboxyl group, or to a reactive group on an amino acid side chain such as a sulfur, an amino, a hydroxyl, or a carboxyl moiety. Many fluorophores normally contain suitable reactive sites. Alternatively, the fluorophores may be derivatized to provide reactive sites for linkage to another molecule. Fluorophores derivatized with functional groups for coupling to a second molecule are commercially available from a variety of manufacturers. The derivatization may be by a simple substitution of a group on the fluorophore itself, or may be by conjugation to a linker. Various linkers are well known to those of skill in the art and are discussed below.

As indicated above, in a preferred embodiment, the fluorophores are directly linked to the peptide backbone of the protease indicator. Thus, for example, the 5'-carboxytetramethylrhodamine (5-TMR) fluorophore may be linked to aspartic acid via the alpha amino group of the amino acid as shown in Formula **V**. The iodoacetamide group of rhodamine X acetamide (R492)) may be linked by reaction with the sulfhydryl group of a cysteine as indicated in formula **VI**. Means of performing such couplings are well known to those of skill in the art, and the details of one such coupling are provided in Example 1.

One of skill in the art will appreciate that when the peptide spacers (S^1 or S^2) are present (as is discussed below), the fluorophores are preferably linked to the conformation determining regions through a reactive group on the side chain of the terminal amino acid of C^1 or C^2 as the spacers themselves form a peptide linkage with the terminal amino and carboxyl groups of C^1 or C^2 respectively.

Selection of spacer peptides and linkage to a solid support

The fluorogenic protease indicators of the present invention may be obtained in solution or linked to a solid support. a "solid support" refers to any solid material that does not dissolve in or react with any of the components present in the solutions utilized for assaying for protease activity using the fluorogenic protease indicator molecules of the present invention and that provides a functional group for attachment of the fluorogenic molecule. Solid support materials are well known to those of skill in the art and include, but are not limited to silica, controlled pore glass (CPG), polystyrene, polystyrene/latex, carboxyl modified teflon, dextran, derivatized polysaccharides such as agar bearing amino, carboxyl or sulfhydryl

groups, various plastics such as polyethylene, acrylic, and the like. Also of use are "semi-solid" supports such as lipid membranes as found in cells and in liposomes. One of skill will appreciate that the solid supports may be derivatized with functional groups (*e.g.* hydroxyls, amines, carboxyls, esters, and sulfhydryls) to provide reactive sites for the attachment of linkers or the direct attachment of the peptide.

The fluorogenic protease indicators may be linked to a solid support directly through the fluorophores or through the peptide backbone comprising the indicator. Linkage through the peptide backbone is most preferred.

When it is desired to link the indicator to a solid support through the peptide backbone, the peptide backbone may comprise an additional peptide spacer (designated S^1 or S^2 in Formula I). The spacer may be present at either the amino or carboxyl terminus of the peptide backbone and may vary from about 1 to about 50 amino acids, more preferably from 1 to about 20 and most preferably from 1 to about 10 amino acids in length. Particularly preferred spacers include Asp-Gly-Ser-Gly-Gly-Gly-Glu-Asp-Glu-Lys, Lys-Glu-Asp-Gly-Gly-Asp-Lys, Asp-Gly-Ser-Gly-Glu-Asp-Glu-Lys, and Lys-Glu-Asp-Glu-Gly-Ser-Gly-Asp-Lys.

The amino acid composition of the peptide spacer is not critical as the spacer just serves to separate the active components of the molecule from the substrate thereby preventing undesired interactions. However, the amino acid composition of the spacer may be selected to provide amino acids (*e.g.* a cysteine or a lysine) having side chains to which a linker or the solid support itself, is easily coupled. Alternatively the linker or the solid support itself may be attached to the amino terminus of S^1 or the carboxyl terminus of S^2.

In a preferred embodiment, the peptide spacer is actually joined to the solid support by a linker. The term "linker", as used herein, refers to a molecule that may be used to link a peptide to another molecule, (*e.g.* a solid support, fluorophore, *etc.*). a linker is a hetero or homobifunctional molecule that provides a first reactive site capable of forming a covalent linkage with the peptide and a second reactive site capable of forming a covalent linkage with a reactive group on the solid support. The covalent linkage with the peptide (spacer) may be via either the terminal carboxyl or amino groups or with reactive groups on the amino acid side-chain (*e.g.* through a disulfide linkage to a cysteine).

Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. As indicated above, the linkers may be joined to the carboxyl and amino terminal amino acids through their terminal carboxyl or amino groups or through their reactive side-chain groups.

Particularly preferred linkers are capable of forming covalent bonds to amino groups, carboxyl groups, or sulfhydryl. Amino-binding linkers include reactive groups such as carboxyl groups, isocyanates, isothiocyanates, esters, haloalkyls, and the like. Carboxyl-binding linkers are capable of forming include reactive groups such as various amines, hydroxyls and the like. Finally, sulfhydryl-binding linkers include reactive groups such as sulfhydryl groups, acrylates, isothiocyanates, isocyanates and the like. Particularly preferred linkers include sulfoMBS (m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester) for linking amino groups (*e.g.* an amino group found on a lysine residue in the peptide) with sulfhydryl groups found on the solid support, or vice versa, for linking sulfhydryl groups (*e.g.* found on a cysteine residue of the peptide) with amino groups found on the solid support. Other particularly preferred linkers include EDC (1-ethyl-3-(3-dimethylaminopropryl)-carbodiimide) and bis-(sulfosuccinimidyl suberate). Other suitable linkers are well known to those of skill in the art.

The fluorogenic compounds of the present invention may be linked to the solid support through either the S^1 or the S^2 spacer such that the donor fluorophore is either retained on the solid support after cleavage of the molecule by a protease or such that the donor fluorophore goes into solution after cleavage. In the former case, the substrate is then assayed for fluorescence to detect protease activity, while in the later case the solution is assayed for fluorescence to detect protease activity.

Detection of Protease Activity

The present invention also provides methods for utilizing the fluorogenic protease indicators to detect protease activity in a variety of contexts. Thus, in one embodiment, the present invention provides for a method of using the fluorogenic indicators to verify or quantify the protease activity of a stock solution of a protease used for experimental or industrial purposes. Verification of protease

activity of stock protease solutions before use is generally recommended as proteases often to loose activity over time (*e.g.* through self-hydrolysis) or to show varying degrees of activation when activated from zymogen precursors.

Assaying for protease activity of a stock solution simply requires adding a quantity of the stock solution to a fluorogenic protease indicator of the present invention and measuring the subsequent increase in fluorescence or decrease in exciton band in the absorption spectrum. The stock solution and the fluorogenic indicator may also be combined and assayed in a "digestion buffer" that optimizes activity of the protease. Buffers suitable for assaying protease activity are well known to those of skill in the art. In general, a buffer will be selected whose pH corresponds to the pH optimum of the particular protease. For example, a buffer particularly suitable for assaying elastase activity consists of 50 mM sodium phosphate, 1 mM EDTA at pH 8.9. The measurement is most easily made in a fluorometer, and instrument that provides an "excitation" light source for the fluorophore and then measures the light subsequently emitted at a particular wavelength. Comparison with a control indicator solution lacking the protease provides a measure of the protease activity. The activity level may be precisely quantified by generating a standard curve for the protease/indicator combination in which the rate of change in fluorescence produced by protease solutions of known activity is determined.

While detection of the fluorogenic compounds is preferably accomplished using a fluorometer, detection may by a variety of other methods well known to those of skill in the art. Thus for example, since the fluorophores of the present invention emit in the visible wavelengths, detection may be simply by visual inspection of fluorescence in response to excitation by a light source. Detection may also be by means of an image analysis system utilizing a video camera interfaced to an digitizer or other image acquisition system. Detection may also be by visualization through a filter as under a fluorescence microscope. The microscope may just provide a signal that is visualized by the operator. However the signal may be recorded on photographic film or using a video analysis system. The signal may also simply be quantified in real-time using either an image analysis system or simply a photometer.

Thus, for example, a basic assay for protease activity of a sample will involve suspending or dissolving the sample in a buffer (at the pH optima of the particular protease being assayed), adding to the buffer one of th fluorogenic protease indicators of the present invention, and monitoring the resulting change in fluorescence using a spectrofluorometer. The spectrofluorometer will be set to excite the donor fluorophore at the excitation wavelength of the donor fluorophore and to detect the resulting fluorescence at the emission wavelength of the donor fluorophore.

In another embodiment, the protease activity indicators of the present invention may be utilized for detection of protease activity in biological samples. Thus, in a preferred embodiment, this invention provides for methods of detecting protease activity in isolated biological samples such as sputum, blood, blood cells, tumor biopsies, and the like, or *in situ*, in cells or tissues in culture, or in section where the section is unimbedded and unfixed. The signal may be quantified using a fluorescence microscope, a fluorescence microplate reader, a fluorometer, or a flow cytometer.

***Ex vivo* assays of isolated biological samples**

In one embodiment, the present invention provides for methods of detecting protease activity in an isolated biological sample. This may be determined by simply contacting the sample with a fluorogenic protease indicator of the present invention and monitoring the change in fluorescence of the indicator over time. The sample may be suspended in a "digestion buffer" as described above. The sample may also be cleared of cellular debris, *e.g.* by centrifugation before analysis.

Where the fluorogenic protease indicator is bound to a solid support the assay may involve contacting the solid support bearing the indicator to the sample solution. Where the indicator is joined to the solid support by the side of the molecule bearing the donor fluorophore, the fluorescence of the support resulting from digestion of the indicator will then be monitored over time by any of th means described above. Conversely, where the acceptor molecule fluorophore is bound to a solid support, the test solution may be passed over the solid support and then the resulting luminescence of the test solution (due to th cleaved

fluorophore) is measured. This latter approach may be particularly suitable for high throughput automated assays.

***In situ* assays of histological sections**.

In another embodiment, this invention provides for a method of detecting *in situ* protease activity in histological sections. This method of detecting protease activity in tissues offers significant advantages over prior art methods (*e.g.* specific stains, antibody labels, *etc.*) because, unlike simple labeling approaches, *in situ* assays using the protease indicators indicate actual activity rather than simple presence or absence of the protease. Proteases are often present in tissues in their inactive precursor (zymogen) forms which are capable of binding protease labels. Thus traditional labeling approaches provide no information regarding the physiological state, *vis à vis* protease activity, of the tissue.

The *in situ* assay method generally comprises providing a tissue section (preferably a frozen section), contacting the section with one of the fluorogenic protease indicators of the present invention, and visualizing the resulting fluorescence. Visualization is preferably accomplished utilizing a fluorescence microscope. The fluorescence microscope provides an "excitation" light source to induce fluorescence of the "donor" fluorophore. The microscope is typically equipped with filters to optimize detection of the resulting fluorescence. Thus, for example, for the fluorogenic protease indicators described in Example 1, a typical filter cube for a Nikon microscope would contain an excitation filter (λ=550 ± 12 nm), a dichroic mirror (λ=580 nm) and an interference-emission filter (λ=580 ± 10 nm). As indicated above, the microscope may be equipped with a camera, photometer, or image acquisition system.

The sections are preferably cut as frozen sections as fixation or embedding will destroy protease activity in the sample.

The fluorogenic indicator may be introduced to the sections in a number of ways. For example, the fluorogenic protease indicator may be provided in a buffer solution, as described above, which is applied to the tissue section. Alternatively, the fluorogenic protease indicator may be provided as a semi-solid medium such as a gel or agar which is spread over the tissue sample. The gel helps to hold moisture in the sample while providing a signal in response to

protease activity. The fluorogenic protease indicator may also be provided conjugated to a polymer such as a plastic film which may be used in procedures similar to the development of Western Blots. The plastic film is placed over the tissue sample on the slide and the fluorescence resulting from cleaved indicator molecules is viewed in the sample tissue under a microscope.

Typically the tissue sample must be incubated for a period of time to allow the endogenous proteases to cleave the fluorogenic protease indicators. Incubation times will range from about 10 to 60 minutes at temperatures up to and including 37°C.

In situ Assays of cells in culture and Cell Suspensions Derived from Tissues and Biopsy Samples.

In yet another embodiment, this invention provides for a method of detecting *in situ* protease activity of cells in culture or cell suspensions derived from tissues, biopsy samples, or biological fluids (*e.g.*, saliva, blood, urine, lymph, plasma, *etc.*). The cultured cells are grown either on chamber slides or in suspension and then transferred to histology slides by cytocentrifugation. Similarly, the cell suspensions are prepared according to standard methods and transferred to histology slides. The slide is washed with phosphate buffered saline and coated with a semi-solid polymer or a solution containing the fluorogenic protease indicator. The slide is incubated at 37°C for the time necessary for the endogenous proteases to cleave the protease indicator. The slide is then examined under a fluorescence microscope equipped with the appropriate filters as described above.

Alternatively, the cells are incubated with the protease indications at 37°C, then washed with buffer and transferred to a glass capillary tube and examined under a fluorescence microscope. When a flow cytometer is used to quantitate the intracellular enzyme activity, the cells with the fluorogenic indicator is simply diluted with buffer after 37°C incubation and analyzed.

Other Indicat r Compositions.

As explained above, it was a discovery of this invention that fluorescent molecules covalently attached on opposite sides of a backbone (*e.g.*,

peptide cleavage site) can quench by self-int raction (*e.g.*, through the formation of dimers). Thus, in one embodiment, indicator molecules can be made using a single fluorophore rather than a matched donor-acceptor pair. Also, as explained above, particularly preferred fluorophores are those that form H-type dimers (*e.g.*, carboxytetramethylrhodamine, carboxyrhodamine-X, diethylaminocoumarin and carbocyanine dyes).

The use of single species labeled indicators, however, is not restricted to peptide-based compositions. To the contrary,"homo-double labeled" indicator molecules can utilize a variety of backbones including, but not limited to nucleic acid backbones, oligosaccharide backbones, lipid backbones, and the like. Methods of coupling fluorophores to such backbones are well known to those of skill in the art. For example, conjugation methods for attaching fluorophores to amino acids, peptides, proteins, nucleic acids, oligonucleotides, sugars , polysaccharides, proteoglycans, lipids, glycolipids and lipopolysaccharides, are described by Hermanson, (1995) *Bioconjugate Techniques*, Academic Press New York, N.Y., Kay M. *et al.*, (1995) *Biochemistry*, 34: 293-300, and by Stubbs, *et al.* (1996) *Biochemistry* 35: 937- 947.

Nucleic acid indicators

Homo-doubly labeled nucleic acid backbones provide effective indicators for nucleic acid hybridizations and/or endonuclease activity. In this embodiment, a nucleic acid backbone is labeled with a self-quenching (*e.g.*, H-type dimer-forming) fluorophore at the 3' and 5' end (either through a direct attachment or indirectly through (*e.g.*, a peptide) linker). The nucleic acid backbone is selected to include self-complementary regions and thereby form a hairpin or other self-hybridized conformation that brings the fluorophores into proximity so that self-quenching occurs. When the indicator (probe) thus formed is hybridized to a complementary target nucleic acid, the self-hybridization is eliminated, the fluorophores are separated and the fluorescence signal produced by the molecule increases. Alternatively, the fluorescently labeled nucleic acid backbone can be used to assay for nuclease activity (*e.g.*, restriction endonuclease or ribozyme activity). When the nucleic acid backbone is cleaved by a nuclease (*e.g.*, by restriction endonuclease recognition of a target site in the backbone) the

fluorophores are separated again increasing the fluorescence signal. Methods of selecting appropriate nucleic acid backbones are d scribed by Tyagi and Kramer *et al.* (1996) *Nature Biotechnology*, 14: 303-308.

The homo-doubly labeled fluorescently DNA probes can be used for detection, localization, or quantification of target DNA sequences in a variety of contexts. Thus, for example, the nucleic acid indicators of this invention can be used for rapid detection of amplification products in nucleic acid amplification (*e.g.*, PCR) reactions. Here the indicator is selected with a backbone complementary to a region of the amplification product. As amplification product is produced the indicator hybridizes to the product and the fluorescence signal activity of the PCR solution increases. The nucleic acid indicators can be used as hybridization or nuclease activity indicators in a variety of other contexts. For example, in *in situ* hybridization (*e.g.*, FISH) mapping of genomic DNA sequences can be accomplished using fluorescent probes to target particular regions within chromosomes (*see, e.g.*, Meyne(1993) *Chromosome mapping by fluorescent in situ hybridization,* pp 263-268 In: *Methods in Nonradioactive Detection* G.C. Howard, ed., Appleton & Lange, Norwalk, Connecticut; Morrison (1992) *Detection of energy transfer and fluorescence quenching,* pp. 311-352 In: *Nonisotopic DNA Probes Techniques* L.J. Kricka, ed. Academic Press, New York; and Varani (1995) *Annu. Rev. Biophys. Biomol. Struct.* 24: 379-404).

In another embodiment, the self-quenching fluorophores can be used to assay two molecule interactions (*e.g.*, protein-protein, protein-nucleic acid, ligand -receptor, *etc.*). In this embodiment, one fluorophore is attached to one molecule (*e.g.*, a protein) while the second fluorophore is attached to a second molecule (*e.g.*, a second nucleic acid or a nucleic acid binding protein). When the two molecules bind, the fluorophores are juxtaposed and quench each other (*e.g.*, through the formation of H-type dimers). The use of donor-acceptor resonance energy transfer systems to measure two molecule interactions is described by Bannwarth *et al.*, *Helvetica Chimica Acta.* (1991) 74: 1991-1999, Bannwarth *et al.* (1991), *Helvetica Chimica Acta.* 74: 2000-2007, and Bannwarth *et al.*, European Patent Application No. 0439036A2.

Olig saccharid Indicators

Homo-doubly labeled oligosaccharide backbone indicators permit the detection of glycosidase activity and lecithin binding protein identification. The fluorophores can be conjugated directly to an oligosaccharide or glycopeptide backbone or attached indirectly through (*e.g.*, peptide) linkers. The oligosaccharides and/or glycopeptides can be chemically synthesized, recombinantly expressed, or isolated from natural sources such as fetuin and other glycoproteins by proteolytic fragmentation of the parent glycoproteins.

As in the case for oligonucleotides, an oligosaccharide specific structure may be selected for detection of a specific glycosidase, an enzyme that hydrolyzes bonds between two sugar molecules.

When a specific oligosaccharide or lecithin is selected to look for its lecithin binding protein, then the increased fluorescence indicates the complexation events that disrupt the H-type dimer, either by separating two dyes or distorting the relative orientation of two dyes. These effects result in increased fluorescence from the homo-double labeled probe.

Lipid Indicators

When a lipid, glycolipid or lipopolysaccharide are labeled with a self-quenching (*e.g.*, H-type dimer forming) fluorophore and added to liposomes or other lipid (*e.g.*, biological) membranes, a decrease in fluorescence will indicate H-type dimer formation and the degree of such fluorescence intensity will be an indication of the amount of H-type dimer formation. Because of the relative fluidity of a lipid membrane, the self-quenching fluorophores are able to interact (*e.g.* approach to a spacing of about 6 to about 10 Å) a stable H-type dimer results. When a membrane active agent, for example, an agent that affects either membrane fluid dynamics or permeabilization to a test compound, is added, then the observed fluorescence intensity changes indicate the test compound's ability to modify membrane fluidity or permeabilization. Hence, such labeled lipids are useful in drug screening and in development of lipid-drug delivery vehicles.

Similarly, the lipid-based probes of this invention can be used to similarly investigate the degree of lipid/protein interaction.

Cellular Uptake of P lypeptides.

It was also a discovery of this invention that attachment of a hydrophobic protecting group to a polypeptide enhances uptake of that polypeptide by a cell.. The effect is most pronounced when the polypeptide also bears a fluorophore, more preferably two fluorophores (*see*, Example 9). Preferred hydrophobic groups include, but are not limited to Fmoc, benzyloxycarbonyl, Xanthyl (Xan), Trityl (Trt), 4-methyltrityl (Mtt), 4-methoxytrityl (Mmt), 4-methoxy-2,3,6-trimethyl-benzenesulphonyl (Mtr), Mesitylene-2-sulphonyl (Mts), 4,4'-dimethoxybenzhydryl (Mbh),Tosyl (Tos), 2,2,5,7,8-pentamethyl chroman-6-sulphonyl (Pmc), 4-methylbenzyl (MeBzl), 4-methoxybenzyl (MeOBzl), Benzyloxy (BzlO), Benzyl (Bzl), Benzoyl (Bz), 3-nitro-2-pyridinesulphenyl (Npys), 1-(4,4-dimentyl-2,6-diaxocyclohexylidene)ethyl (Dde), 2,6-dichlorobenzyl (2,6-DiCl-Bzl), 2-chlorobenzyloxycarbonyl (2-Cl-Z), 2-bromobenzyloxycarbonyl (2-Br-Z), Benzyloxymethyl (Bom), t-butoxycarbonyl (Boc), cyclohexyloxy (cHxO),t-butoxymethyl (Bum), t-butoxy (tBuO), t-Butyl (tBu), Acetyl (Ac), and Trifluoroacetyl (TFA).

This invention thus provides a method of delivering a molecule (*e.g.*, a polypeptide, oligonucleotide, oligosaccharide, a lipid, *etc.*) into a cell. The method involves providing the molecule to be delivered (*e.g.*, polypeptide) having attached at least two fluorophore molecules and a hydrophobic group, more preferably an Fmoc group and then contacting the cell with the molecule.

It will be appreciated that where the peptide, oligonucleotide, oligosaccharide, or lipid is to be delivered *in vivo* for diagnostic end point or for therapeutic purposes, fluorophores and a hydrophobic group having reduced or no toxicity are preferred.

Thus, in a preferred embodiment, the fluorophores are replaced with non-toxic molecules having little or no biological activity. Preferred molecules are fused ring compounds that act as a linker joining the two ends of the molecule that is to be delivered. Particularly preferred fused ring compounds approximate the spacing of the exciton dimer.

Most preferred fused ring compounds include, but are not limited to steroids. The relatively flat and hydrophobic fluorophores that are known for H-type dimer formation can be replaced with similarly hydrophobic and structurally rigid

and/or flat fused rings found, for example, in steroid molecules. a steroid derivative, *e.g.*, a smaller than full steroid molecule, two to three fused six member ring molecules can be cross linked via usual cross linkers to provide a size and an over all hydrophobicity comparable to the Fmoc and other hydrophobic groups described herein. Since safe metabolic pathways exist for larger molecule consisting of these smaller building blocks, the toxicity of such hybrid molecules is expected to be small. In a preferred embodiment, the hydrophobic molecules are in a size range of about 17 by 12 Angstroms. It will be appreciated that where the peptide is to be delivered *in vivo* fluorophores of reduced or no toxicity are preferred. Toxicities of numerous fluorophores are well known to those of skill in the art (*see, e.g.*, Haugland, *Handbook of Fluorescent Probes and Research Chemicals, 6th Ed.*, Molecular Probes, Eugene, OR. (1996). In addition, toxicity (*e.g.*, LD_{50}) can be readily determined according to standard methods well known to those of skill in the art. In a most preferred embodiment, the fused ring compound is a fused steroid such as structures XI and XII illustrated in Latt *et al.*(1965) *J. Am. Chem. Soc.*, 87: 995-1003, where $-OR_1$ and $-OR_2$ can serve as activated points of attachment for the ends of peptides, nucleic acids or other molecules it is desired to transport into the cell.

As indicated above, the cellular uptake of almost any molecule will be enhanced by the attachment of the hydrophobic group and fluorophore or steroid cross-linkers. Thus, suitable molecules include virtually any molecule it is desired to introduce into the cell. Particularly preferred molecules include, but are not limited to, polypeptides (*e.g.*, the protease inhibitors of this invention) and nucleic acids (*e.g.* oligonucleotide HIV inhibitors (*see, e.g.*, Jing (1997) *Biochem.*, 36: 12498-12505), ribozymes, peptide nucleic acids, and the like).

Activity Detection Kits

The present invention also provides for kits for the detection of protease activity in samples. The kits comprise one or more containers containing the fluorogenic protease indicators of the present invention. The indicators may be provided in solution or bound to a solid support. Thus the kits may contain indicator solutions or indicator "dipsticks", blotters, culture media, and the like. The kits may also contain indicator cartridges (where the fluorogenic indicator is bound

to the solid support by the "acceptor" fluorophore side) for use in automated protease activity detectors.

The kits additionally may include an instruction manual that teaches the method and describes use of the components of the kit. In addition, the kits may also include other reagents, buffers, various concentrations of protease inhibitors, stock proteases (for generation of standard curves, etc), culture media, disposable cuvettes and the like to aid the detection of protease activity utilizing the fluorogenic protease indicators of the present invention.

It will be appreciated that kits may additional or alternatively comprise any of the other indicators described herein (*e.g.*, nucleic acid based indicators, oligosaccharide indicators, lipid indicators, *etc*). In this instance the kit will facilitate detection of the particular activities/compounds/interactions for which the particular indicator backbone is a substrate or binding agent.

Protease Inhibitors

It was also a discovery of this invention that the protease indicators can also act as protease inhibitors. Protease inhibitors and protease substrates share several basic properties such as ability to bind to protease's catalytic substrate binding site, and form a relatively stable complex with a protease. Hence, many normal substrates or their fragments exhibit competitive substrate inhibition at higher concentrations. The inhibition is competitive since the inhibitor binds to the same substrate binding site of the protease whereby it competes with the native substrate in binding to the protease's catalytic domain.

This invention provides three novel approaches for protease inhibitor design. In the first approach, a normal substrate is redesigned such that it binds to protease well, but has a reduced (slow or non-existent) hydrolysis rate. The slow hydrolysis rate is achieved by introducing an altered (different) conformation and/or conformational flexibility into the protease recognition domain. After the (*e.g.*, native) substrate binds to the protease's substrate binding site, the conformation of the peptide bond between P_1 and P_1' is distorted into a transition conformation of a given protease's peptide bond hydrolysis reaction. If this peptide bond as well as adjacent peptide bonds are altered such that they are not distortable then the hydrolysis rate will be reduced as compared to a substrate whose cleavage site

peptide bond is easily distorted into the desired transition conformation. This approach is illustrated in Example 16 which shows how one can vary the hydrolysis rate of a substrate without changing the protease recognition amino acid sequences.

In a second approach, the inhibitor is produced by replacing the critical P_1 or P_1' residue which makes it difficult to distort the cleavage site peptide bond. Normally, the amino acid side chains of P_1 and P_1' residues interact specifically with the side chains of the protease catalytic domain. These specific interaction facilitate coordination of the peptide bond distortion into a transition conformation of the hydrolysis reaction. Thus, for example, when the critical P_1 residue of aspartic acid residue in the CPP32 protease substrate is replaced with non-charged asparagine then normal interaction between the substrate and protease does not take place even though the modified substrate binds to the protease's substrate binding site. Again, this leads to a slower or zero hydrolysis rate. The example of this P_1 residue substitution effect in designing an inhibitor is illustrated by the properties of the DEVN peptide (*see, e.g.,* Figure 5 and Example 12). The biological conformation that the substrate DEVN is an inhibitor is demonstrated in Example 13. Additional evidence that the peptide DEVN does bind to protease is given in Example 15.

The P_1' residue can be changed to introduce either charged amino acid side chains or a structurally rigid, *e.g.,* proline, residue as illustrated in the Table 3, substrate sequences for Hepatitis C viral protease substrate NS3 NS5A/5B of DVVCCSMS (normal substrate) and DVVCCPdMS (inhibitor). The underlined residues are the P_1 residues.

In a third approach, the amide bond between P_1 and P_1' residues of a substrate can be changed to a non-hydrolyzable chemical bond including, but not limited to an ether, thioether, methylene bond, or alkylene (C=C) or ether bond (C-O-C(=O)) keeping the same amino acid side chains for the P_1 and P_1' residues. Also the amide bond can be substituted with a retroinverso bond or other pseudoamino acid bond such as CH_2-NH or C(=O)-S replacing the carbonyl group with a CH_2 group.

EXAMPLES

The invention is illustrated by the following examples. These examples are offered by way of illustration, not by way of limitation.

Example 1

Synthesis of Fluorogenic Molecule for Detecting Protease Activity

a) Synthesis of the peptide backbone.

The amino acid sequences Asp-Ala-Ile-Pro-Nle-Ser-Ile-Pro-Cys (where C^1 is Asp-Ala-Ile, P is Pro-Nle-Ser-Ile and C^2 is Pro-Cys) and Asp-Ala-Ile-Pro-Met-Ser-Ile-Pro-Cys (where C^1 is Asp-Ala-Ile, P is Pro-Met-Ser-Ile and C^2 is Pro-Cys) were synthesized manually utilizing a t-Boc Cys-Pam resin and t-Boc chemistry using the protocol for a coupling cycle given below in Table 5. The synthesized peptides were deprotected by treatment with hydrofluoric acid for 60 minutes under anhydrous conditions at a temperature of 4°C.

Table 5. Reaction steps for one coupling cycle (addition of one amino acid) for the synthesis of the peptide backbone of the fluorogenic protease inhibitors.

Step	Process	Time (min)	# Repeats
1	TFA/DCM Pre-wash 15 ml, 50% (v/v) or 35% (v/v)	2.0	1
2	TFA/DCM Main Wash 15 ml, 50% (v/v) or 35% (v/v)	28.0	1
3	DCM washes, 10 ml	<0.33	4
4	DIEA/DCM, 10% (v/v), 10 ml	2.0	1
5	DIEA/DCM, 10% (v/v), 10 ml	8.0	1
6	DCM washes, 10 ml	<0.33	5
7	Resin wash with NMP (or DMF)	<0.33	5
8	5-fold excess t-Boc-amino acid with HOBT and DIPC in NMP or DMF	90 to 120	1
9	DCM wash	<0.33	1
10	MeOH wash	<0.33	1
11	DCM washes	<0.33	2
12	DIEA wash	<0.33	1
13	DCM washes	<0.33	3
14	Ninhydrin	5.0	1
15	3-fold excess t-Boc-amino acid/DIPC/DCM	30	1

16	MeOH wash	<0.33	1
17	DCM washes	<0.33	5
18	Ninhydrin test	5	1
19	Go to step 1 to couple next amino acid		

Crude post-HF deprotected and cleaved peptides were purified by reverse phase HPLC using a preparative C_{18} column (YMC, Inc., Charlestown, North Carolina, USA). The solvent system utilized was water and acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). HPLC was performed using the following gradient at a flow rate of 10 ml/minute:

Table 6. HPLC gradient for purification of the peptide backbone.

Time (min)	% Solvent a (H_2O with TFA (0.1%)
0	100
7	100
8	90
68	50
78	50

Purification of methionine-containing peptides required subjecting the peptide to reducing conditions, *e.g.*, dithiothreitol (DTT) and heat, to reduce methionine oxide to methionine. This reductive treatment was carried out by dissolving the peptide in 150 mM sodium phosphate buffer with 1 mM DTT, pH 7.5 for 30 minutes at 60 to 80°C. The reduction could also have been carried out under a weak acidic pH with 0.03 N HCL but would have required a longer heating time. The subsequently HPLC purified methionine containing peptides were found to oxidize upon sitting either in aqueous solution or lyophilized form. The oxidized peptides were found to be reducible repeating the above reducing condition.

b) Derivatization of the peptide backbone with the fluorophore molecules.

The peptides were derivatized sequentially with donor and acceptor fluorophores. Specifically the donor fluorophore (5'-carboxytetramethylrhodamine (5-TMR), available from Molecular Probes, Inc. Eugene, Oregon, USA) was first covalently linked to the amino terminus of the peptide. The peptide and the probe,

at a molar ratio of 3:1 peptide to probe, were dissolved in a minimal amount of solvent NMP (N-methyl pyrolidone), usually 20 to 60 µl. One molar equivalent of DIEA (diisopropyl ethyl amine) was also added to the reaction mixture. The reaction was then incubated at 37°C with times ranging between 12 hours and 3 days. After two days an additional 1 molar equivalent of dye molecule was sometimes added. The derivatization reached nearly maximal yield by 3 days. The peptide bearing the single 5-TMR fluorophore was then HPLC purified as described below.

The second (acceptor) fluorophore (rhodamine X acetamide (R492)) was then coupled to the carboxyl cysteine of the peptide by a linkage between the iodoacetamide group of the fluorophore and the sulfhydryl group of the terminal cystein. This coupling was accomplished as described above for the first fluorophore.

The complete fluorogenic protease indicator was then purified by HPLC using an analytical reverse phase C_3 columns (2 ml void volume) from Waters Associates Inc. (Milford, Massachusetts, USA) using the gradient shown in Table 6 running at 1 ml/minute.

Table 7. HPLC gradient for purification of the peptide bearing fluorophores.

Time (min)	% Solvent a (H_2O with TFA (0.1%)
0	100
1	100
2	80
6	80
66	50

The slow reactivity of both the amino and sulfhydryl groups in coupling the fluorophores appeared to be a function of the folded structure of the peptide backbone which sterically hindered access to the peptide's reactive groups. Control experiments using irrelevant linear peptides showed considerably faster linking. The folded structure is also supported by the results reported in Examples 2 and 3. a computer energy minimization model of the peptide also indicated possible preference for the peptide to assume a folded structure rather than an open extended structure. This is due largely to the presence of the conformation determining regions containing the two proline residues.

Exampl 2

Alternative Synth sis f Proteas Activity Indicators

a. Fmoc-protected peptide backbone synthesis

The amino acid sequences listed in Table 8 were synthesized manually utilizing Fmoc chemistry and 2-chlorotritylchloride resin employing the protocol for a coupling cycle given below in Table 7.

Table 8. Protease indicator peptide backbones.

	S^1	C^1	P	C^2	S^2
1.		Asp-Ala-Ile-	Pro-Nle-Ser-Ile-	Pro-Cys-	Gly-Tyr
2.		Asp-Ala-Ile-	Pro-Nle-Ser-Ile-	Pro-Lys-	Gly-Tyr
3.	Lys-	Asp-Ala-Ile-	Pro-Nle-Ser-Ile-	Pro-Lys-	Gly-Tyr
4.		Asp-Aib-Thr-	Gly-Arg-Thr-Gly-	Pro-Lys-	Gly-Tyr
5.	Lys-	Asp-Aib-Thr-	Gly-Arg-Thr-Gly-	Pro-Lys-	Gly-Tyr

The synthesized peptides were cleaved from the 2-chlorotritylchloride resin with mild acid treatment (vol/vol ratio of 2:7:1 Acetic acid: Dichloromethane: Trifluoroethanol) at room temperature for 30 minutes. a 10 ml aliquot of this peptide resin cleavage solution was added to 0.1 gm of dried peptide resin. The following side chain protecting groups were used in the synthesis: t-butyl for Asp, Ser, Thr, and Tyr residues, S-trityl and S-t-butylthio for Cys residues, and t-Boc, Fmoc and 4-methyltrityl for lysine residues.

The side chain protecting groups as well as the Fmoc group on the alpha amino group of the synthesized peptide were not cleaved with this mild acid peptide resin cleavage reagent. The protected peptide containing solution was lyophilized. The lyophilized protected peptides were further treated by either 30% (v/v) TFA in dichloromethane for t-Boc deprotection, 20%(v/v) piperidine in DMF or N-methylpyrolidone for Fmoc deprotection, 1 to 5% (v/v) trifluoroacetic acid (TFA) in water, or 1% TFA/5% triisopropylsilane in DCM, for 4-methyltrityl deprotection, aqueous mercaptoethanol (10%) for S-t-Butylthio deprotection, TFA : phenol : water : thioanisol : ethanedithiol=85 : 5 : 5 : 2.5 : 2.5 for t-Butyl, t-Boc and S-Trityl deprotection, and TFA:phenol:water=90 : 5 : 5 for t-Butyl and t-Boc deprotection.

Fully or partially side-chain deprotected peptides were purified by reversed phase HPLC using a C18 column with a water/acetonitrile gradient containing 0.075% (v/v) TFA in each solvent.

b. Derivatization of the protected peptide backbone with fluorophores.

The fully purified protected peptide was further treated with the appropriate reagents for selective deprotection of the side chains of Cys or Lys residues. The use of three different protecting groups, *i.e.*, t-Boc , Fmoc, and 4-methyltrityl group, for the epsilon (ϵ) amino group protection of lysine, allowed selective deprotection, and thus, selective derivatization of a specific lysine residue.

For example, about 1 mg of protected peptide was dissolved in a minimal amount of N-Methylpyrolidone. The appropriate fluorophore derivatized with a succimidyl ester reactive functional group was added to the peptide solution at a 1.2 to 2 fold molar excess of reactive fluorophore over the peptide. a ten-fold mole excess of diisopropylethylamine (DIEA) was also added to the reaction mixture. The reaction was allowed to proceed at room temperature for 2 to 4 hours. The derivatized peptides were purified by reversed phase HPLC using a C18 or C4 column and 0.075% (v/v) TFA-containing water/acetonitrile solvent system.

The derivatization of the peptide with the first fluorophore was facilitated by the presence of at least one very hydrophobic group such as Fmoc. The presence of such a hydrophobic group on the peptide allowed elution (*e.g.* partitioning) of the derivatized peptide away from fluorophore contaminants and reaction by-products and degradation products that accumulate as the derivatization reaction is allowed to proceed.

Deprotection of the Fmoc group was then carried out after one amino group or sulfhydryl group was derivatized with the desired fluorophore. The fluorophores utilized for the amino group conjugation were 6-TMR, 5-(and 6-)carboxytetramethylrhodamine succinimidyl ester, DER, 5-(and 6-)carboxy-X-rhodamine succinimidyl ester, and fluorescein isothiocyanate. After deprotection, a second fluorophore was added in a manner identical to the addition of the first.

Table 9. Reaction steps for one coupling cycle (addition of one amino acid) for the synthesis of th peptide backbone of the fluorogenic protease substrates.

Step	Process	Time (min)	# repeats
1.	Wash resin with DCM.	1	1
2.	Wash resin with DMF.	1	2
3.	20% piperidine in DMF.	5	1
4.	20% piperidine in DMF.	15	1
5.	Wash resin with DCM.	1	4
6.	Wash resin with NMP.	1	2
7.	Preactivate 2 to 4 fold mole excess Fmoc-aa , PyBoP, HoBt, and N-methylmorpholine in NMP.	5	1
8.	Add the above pre-activated Fmoc-AA coupling mixture.	45	1
9.	Wash resin with DCM.	1	4
10.	Wash resin with methanol.	1	1

c. Molecular weight characterization of the derivatized peptides.

Since during, or after, the derivatization of the protected peptides a strong acid deprotection step was sometimes used to remove the remaining t-Butyl groups from the various amino acid side chains, there was a possibility that either aromatic amino acids or fluorophores might have been chemically modified. The molecular weights of the derivatized and purified peptides were therefore determined.

Molecular weights were measured using a matrix assisted laser desorption time of flight mass spectrometer, Kompact MADLI I by Kratos Analytical. The Mass spectrometer was calibrated with Leucine-Enkaphelin (556.6 amu), Bradykinin (1061.2 amu), and Mellitin (2847.5 amu). The sample matrix used was α-cyano-4-hydroxycinnamic acid. Samples were applied to target and 1 ml 0.1% TFA in ethanol solution was added on the target and then dried down. Cumulative mass spectral data from 50 laser shots were collected and a peak corresponding to the parent mass peak plus 1 for each sample was determined. The results are summarized on Table 10.

The good agreement between the calculated and experimentally determined mass values indicates the absence of any side reaction products in the final purified fluorophore-conjugated peptides.

Table 10. Calculated and determined molecular mass of the derivatized peptide protease substrates. The vertical and horizontal line indicate the fluorophore's attachment site. The symbols FL, 6-TMR and DER denot fluorophores fluorescein isothionate, 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester, and 5-(and-6)-carboxy-X-rhodamine succinimidyl ester, respectively. ND denotes not determined.

	Compound	Calculated Mass (amu)	Observed Mass (amu)
1.	6-TMR-DAIP(Nle)SIPKGY └DER	2101.2	2102.6
2.	DER-DAIP(Nle)SIPKGY └6-TMR	2101.2	2097.8
3.	6-TMR-DAIP(Nle)SIPKGY └6-TMR	1997.0	1997.6
4.	DAIP(Nle)SIPKGY └DER	1688.7	1685.9
5.	DER-DAIP(Nle)SIPKGY	1688.7	1688.1
6.	DAIP(Nle)SIPKGY └6-TMR	1585.5	1585.8
7.	6-TMR-DAIP(Nle)SIPKGY	1585.5	1583.7
8.	Fl-DAIP(Nle)SIPKGY └Fl		ND
9.	DER-DAIP(Nle)SIPKGY └C1309		ND
10.	KDAIP(Nle)SIPKGY 6-TMR┘ └DER		ND
11.	KDAIP(Nle)SIPKGY 6-TMR ┘ └6-TMR		ND

Example 3

The Fluorogenic Protease Indicators Provide a Strong Signal When Digested

In order to demonstrate that the fluorogenic protease indicators of this invention are easily digested by a protease, the degree of cleavage was determined by assaying for the appearance of indicator cleavage products in the presence of a protease.

Approximat ly 1 microgram of protease indicator, having the formula F^1-Asp-Ala-Ile-Pro-Nle-Ser-Ile-Pro-Cys-F^2 wher F^1 is a donor fluorophore (5'-carboxytetramethylrhodamine (5-TMR)) linked to aspartic acid via the alpha amino

group and F^2 is an acceptor fluorophore (rhodamine X acetamide (R492)) linked via the sulfhydryl group of the cysteine was dissolved in a buffer consisting of 50 mM sodium phosphate, 1 mM EDTA at pH 8.9. To this solution was added 1 unit of elastase. The solution was analyzed by HPLC before and about 30 minutes after the addition of elastase. The digestion was carried out at 37°C. The HPLC separated components were monitored at a wavelength of 550 nm which allowed detection of both the 5-TMR fluorophore the R492 fluorophore and at 580 nm which allowed detection of the R492 fluorophore.

The results are indicated in Figure 1 which shows the HPLC profiles of the fluorogenic protease indicator solution before and after addition of the protease elastase. Figure 1(a) shows the HPLC before addition of the elastase showing a single peak representing the intact fluorogenic protease inhibitor. After addition of the elastase (Figures 1(b) and 1(c)) there was no trace of the late eluting single peak (Figure 1(a)) indicating complete digestion of the fluorogenic protease indicator. In addition, the two predominant peaks in Figure 1(b) and 1(c) indicate that the digestion occurred primarily at a single site. There are a few smaller peaks indicating a low degree of digestion at other sites within the peptide sequence, however, the striking predominance of only two digestion peaks suggests that these secondary sites were not readily accessible to the elastase.

Changes in the emission spectrum of the fluorogenic protease indicator after the addition of an elastase protease was monitored using an SLM spectrofluorometer model 48000 with slit widths set at 4 nm on both the excitation and emission sides. All measurements were carried out at 37°C.

Spectra in Figure 2 show emission of the fluorogenic protease indicator (a) before and (b) after addition of elastase, while the time dependent increase of the indicator's donor fluorophore emission intensity, after addition of elastase, is plotted in Figure 3. The fluorogenic protease inhibitor showed more than a 10 fold increase in fluorescence at 589 nm after treatment with the elastase protease (Figure 2(a) compared to Figure 2(b)) with over a 5 fold increase in fluorescence occurring within the first 1000 seconds of exposure to the protease. The changes in intensity between treated and untreated indicators are, to some degree, a function of slit widths used, since they represent the signal integrated across the particular slit width. Thus, if wider slit widths were used (*e.g.* 8 or 16 nm slits) an even greater signal would be provided in response to digestion.

Example 4

The Flu rescenc Signal Was Due to Intram lecular Energy D quenching

In order to show that the fluorescence increase observed after protease treatment was due to intramolecular energy dequenching, the signal produced by elastase digestion of the fluorogenic protease indicator was compared to the signal produced by elastase treatment of the same peptide backbone coupled to either F^1 (5-TMR) or to F^2 (R492). The change in fluorescence intensity of the donor fluorophore after addition of 1 unit of elastase to equal concentrations of the double-fluorophore molecule and the two single-fluorophore molecules.

The results are illustrated in Figure 4. The double-fluorophore molecule showed nearly complete quenching initially, followed by a dramatic increase in fluorescence after addition of the elastase which reached a constant value approximately 30 minutes after addition of the elastase (Figure 4(a)). In contrast, the two single-fluorophore molecules showed virtually no initial quenching and no significant change in fluorescence after addition of the elastase. In fact, the fluorescence level was comparable to the fluorescence level of the fully digested double-fluorophore indicator molecule (Figure 4(b)).

These results indicate that the increase in fluorescence intensity of the fluorogenic protease indicator is due to interruption of the resonance energy transferred intramolecularly from the donor fluorophore to the acceptor fluorophore and not to interaction between the fluorophore and the peptide backbone. This is significant since it is known that upon binding to a large protein or hydrophobic peptide the fluorescence of many hydrophobic fluorophores is quenched.

Example 5

Without being bound to a particular theory, it is believed that the fluorogenic protease indicators of the present invention achieve a high degree of protease specificity due to their folded structure, more particularly due to their relative rigid U-shaped conformation. The fluorescence obtained from the molecule reflects the average separation of two fluorophores. Thus, it was predicted that if the protease indicators existed in a relatively unfolded or flexible state, conditions that tend to cause unfolding (denaturation) would have little or no effect on the fluorescence of the molecule in the absence of a protease. Conversely, if the molecule is relatively rigid, then denaturing conditions would be expected to

increase the fluorescence signal as the average separation of the fluorophores would be expected to increase thereby decreasing the quenching effect.

Thus, the effect of denaturing conditions on the fluorescence of the fluorogenic protease indicator in the absence of a protease was determined. First the change of fluorescence of the indicator of Example 1, as a function of added chaotropic reagent concentration (2M or 8M urea) was measured. When the fluroogenic protease indicator was denatured with a chaotropic reagent the fluorescence intensity increased with time to a plateau as the molecule denatured (unfolded).

These data indicate that the fluorogenic protease indicator normally exists in a stable folded conformation created by the conformation determining regions, as was predicted by a model based on an energy minimization algorithm. The plateau fluorescence level represents residual quenching of the fluorophores still joined by the fully denatured peptide backbone. Digestion of the extended (denatured) peptide results in greater than a 2 fold increase in fluorescence as the fluorophores are able to move farther away from each other.

Example 6

Quenching and Release of a Peptide Doubly-Labeled with One Fluorophore

It was a surprising discovery of this invention that the peptide backbones of this invention doubly labeled with one fluorophore still achieve fluorescence quenching thus suggesting quenching through another mechanism besides resonance energy transfer.

In order to assess the extent ground-state dimerization and collisional quenching contribute to the total observed quenching, the series of doubly-labeled peptides listed in Table 11 was synthesized.

In addition to comparing absorption spectra of the dyes alone with the NorFes peptides singly labeled with each dye, emission spectra taken before and after cleavage were compared to determine the percent of quenching and the existence of fluorescent signal quenching by means other than resonance energy transfer (RET).

Fluorophores were linked to the amino terminus via the α-amino group of Aspartic acid residue (D) and to the ϵ-amino group of lysine (K). Labeling was

accomplished by the displacement of a succinimidyl group linked to 6-TMR or DER. The structure of the peptide, called NorFES-KGY is:

```
Fluorophore1-DAIPNleSIPKGY
                          |
                          |
                          Fluorophore2
```

As determined from absorption spectroscopy, all doubly-labeled peptides, except fluorescein-NorFES-fluorescein, showed the existence of so called ground-state dimers. This was indicated by shift of absorption maxima to shorter wavelengths as well as a shape change of the absorption spectra as compared with the spectra for the enzyme digested doubly-labeled samples. Upon cleavage with elastase, the ground-state dimers were destroyed and the resulting spectra were the same as a solution containing equal concentrations of the respective singly labeled peptides.

Without being bound to a particular theory, it is believed that the ground-state dimer formation observed in the compounds designed and synthesized according to the present invention indicates that the U-shaped conformation of the peptide backbone brings the fluorophores into close spatial proximity thus allowing overlap of electron orbitals of the two fluorophores resulting in reciprocal quenching through ground-state dimerization. It was a surprising discovery that the polypeptides of this invention allowed the formation of ground-state dimers at a significantly lower dye concentration than previously observed. For example, ground-state dimerization of free fluorescein dye in solution was only observed at concentrations higher than 0.74 M, ground-state dimerization of free Eosin dye in solution was only observed at concentrations higher than 2.8×10^{-2} M (*see*, Forster and Konig (1957) *Zeitschrift fur Electrochemie*, 61: 344), and ground-state dimerization of Rhodamine B dye in solution was only observed at concentrations higher than 6×10^{-4} M (see Arbeloa and Ojeda (1982) *Chemical Physics Letters*, 87: 556). In contrast, in the present invention, the effects are observed at 4.0×10^{-7} M or about a 1000 fold lower concentration than the reported values.

The observation of the ground-state dimer for the compounds synthesized according to the present invention predicted a significant level of fluorescent quenching for doubly-labeled peptide with the same fluorophore as those compounds listed in Table 11. In fact this prediction was confirmed; a

comparison of 6-TMR-NorFES-KGY-DER with 6-TMR-NorFES-KGY-6-TMR, *i.e.*, the hetero doubly-labeled with the homo doubly-labeled peptid s, indicates the degree of quenching is slightly higher in the hetero- vs. the homo- (94 vs. 90%). The fluorescein derivative, however, exhibited only 55% quenching. The symbols I_0 and I_c for the percent fluorescent quenching (%Q) refer to the fluorescence intensity for the intact labeled peptide and the enzyme digested labeled peptide solution respectively.

Table 11. Cleavage rate ($T_{1/2}$) and percentage of quenching (%Q) of hetero- and homo-labeled peptides. $T_{1/2}$ is the time in seconds after addition of a protease (*e.g.* elastase) at which the fluorescence signal is 1/2 maximum. The symbols I_o and I_c refer to the fluorescence intensity (I) for the intact labeled peptide and the enzyme digested labeled peptide solution respectively.

Compound	$T_{1/2}$	%Q-(1 - (I_o/I_c)) x 100
6-TMR-NorFes-DER	80	94
6-TMR-NorFes-6-TMR	44	90
6-TMR-NorFes-6-TMR	44	90
DER-NorFes-DER	152	90
F1-NorFes-F1	18	55
6-TMR-NorFes-DER	80	94
6-TMR-K-NorFes-DER	125	97
6-TMR-NorFes-6-TMR	44	90
6-TMR-K-NorFes-6-TMR	84	92

The substrate sequence could be extended by one amino acid residue and the fluorophore could be attached through the episilon amino group on the lysine residue's side chain without major perturbation to the amount of observed quenching. Specifically, this addition (peptides designated K-NorFES-KGY) resulted in a slight decrease in cleavability rate and a very slight increase in the percent quenching for both the hetero- and homo-doubly-labeled peptide (in the K-NorFES-KGY peptides, N-terminal labeling was via the epsilon-amino group of lysine rather than the α-amino terminus).

Rates of cleavage ($T_{1/2}$) of these substrates by elastase were also measured by recording the time after addition of the protease at which the signal was one-half maximum (*see*, Table 11). a comparison of three homo-doubly-labeled peptides, *i.e.*, NorFES-KGY labeled with two molecules of 6-

TMR, DER, and fluorescein (Fl), shows the order of cleavability to be:

Fl-NorFES-KGY-Fl > 6-TMR-NorFES-KGY-6-TMR > DER-NorFES-KGY-DER.

Example 7

Use of Homo-Doubly Labeled Protease Indicators

In order to demonstrate the efficacy of the protease indicators of this invention *in vitro*, cells of the epidermal carcinoma cell line, A431, were grown to incomplete confluence in a Permanox tissue culture chamber slide (Nunc, Inc., Naperville, Illinois, USA) in Dulbecco's Minimal Essential Medium (DME) containing 5% fetal calf serum (FCS). After removal of the medium, 200 µl of a solution containing 20% ethanol was added to each chamber and incubation was carried out for two minutes. The ethanolic medium was then removed and the monolayers were washed twice with DME (minus the FCS).

A DME solution containing 6-TMR-NorFes-6-TMR at a concentration of 1×10^{-7} M was then incubated with the monolayer for 10 minutes. The cells were then examined for fluorescence with a Nikon fluorescence microscope using a rhodamine filter cube. (An advantage of using peptides homo-doubly-labeled with a single fluorophore compared to those labeled with two different fluorophores (hetero-doubly-labeled) is that fluorescence microscopy using homo-doubly-labeled peptides only requires a cutoff filter (*i.e.*, a filter that transmits all light above a defined wavelength) on the emission side of the dichroic mirror, whereas fluorescence microscopy using hetero-doubly-labeled peptides preferably uses an interference filter (*i.e.*, a filter that transmits light in a defined wavelength range (x ± y nm)).

Each cell was clearly defined by a diffuse red fluorescence (produced by the protease indicator cleaved by elastase) filling its entire cytoplasm. For cells at the edge of a confluent island, the black borders of the islands were clearly distinct from the red fluorescence in the cytoplasm of the cells indicating that the fluorescence was not due to background fluorescence or to cleavage of the protease indicator by the medium.

Example 8

In addition, we have synthesized and derivatized (homodoubly-labeled) PAI-2, CS-1 (a 31 residue long peptide) and two DEVD-like peptides that

did not allow the dye-dye dimer formation. The CS-1 peptide shows that in a significantly longer peptide the dye-dye dimer structur can be formed. Note this peptide contains four proline residues in the amino terminal side of the putative cleavage site Ile-Leu bond. There is one proline in the carboxyl domain also. The results from the CS-1 peptide support a potentially larger sequence between the two dyes (fluorophores). Two DEVD-like peptides' amino acid sequences that did not allow the formation of productive H-type dimers are F_1-DEVDGIDPK[F_1]GY and F_1-PDEVDGIDPK[F_1]GY.

Example 9

Cellular Uptake of Substrates Examined by Flow Cytometric and Fluorescence Microscopic Analysis.

The compounds listed in Table 12 were synthesized and assayed for cellular uptake. Cellular internalization of the substrates was tested using Jurkat cells (a human acute T cell leukemic line), HL-60 cells (a human promyelocytic leukemic line), human lymphocyte lines, A1.1 cells (a murine T-cell line), and murine primary thymocytes. Procedures used in determining substrate uptake by viable cells are provided in Example 6 (for the HPLC procedures), in Example 2 (for the fluorescence microscopic analysis), and in Example 3 (for the flow cytometric analysis). a summary of these analyses with respect to cellular uptake of substrates is presented in this example.

Table 12. Compounds assayed for cellular uptake. Abbreviations used in the following table are: F^1: carboxytetramethylrhodamine; Z: benzyloxycarbonyl group; Fm: Fmoc group; K[F1]: F^1 is covalently attached through the epsilon amino group of lysine (K). Single letter amino acid residues are used in the sequences except for Nlu for norleucine, B for aminoisobutyric acid and J for epsilon amino caproic acid residue. H: HPLC, FM: Fluorescence microscopy, FC: flow cytometry.

	Structure	Cellular uptake/ magnitude	Uptake checked by
1	Fm-K[F1] DAIPNluSIPK[F1]GY	Yes/high	FM
2	K[F1] DAIPNluSIPK[F1]GY	Yes/weak	FM
3	Fm-DAIPNluSIPK[F1]GY	No/	FM
4	Fm-K[F1]DBDEVDGIDPK[F1]GY	Yes/high	FM & FC
5	K[F1]DBDEVDGIDPK[F1]GY	Yes/weak	FM
6	Fm-K[F1]DBDEVNGIDPK[F1]GY	Yes/high	FM

7	K[F1]DBDEVNGIDPK[F1]GY	Yes/weak	FM & H
8	Fm-K[F1]DBEVDGIDPK[F1]GY	Yes/high	FM & FC
9	K[F1]DYBADGIDPK[F1]GY	Yes/weak	FM
10	Fm-K[F1]DBGDEVDGIDGPK[F1]GY	Yes/high	H & FC
11	Fm-K[F1]DBJGDEVDGIDGJPK[F1]GY	Yes/high	FC
12	Z-K[F1]DBJGDEVDGIDGJPK[F1]GY	Yes/weak	FM
13	Fm-K[F1]DYBADGIDPK[F1]GY	Yes/high	FM
14	K[F1]DBEVDGIDPK[F1]GY	Yes/weak	FM

The data listed in Table 12 indicate that: (1) the presence of two fluorophores alone is not optimum for cellular uptake as illustrated by structures 2, 5, 7, and 9; (2) addition of a 9-fluorenylmethoxycarbonyl (Fmoc) group at the alpha amino group plus attachment of only one fluorophore, does not result insignificant cellular uptake (*e.g.*, compound 3); and (3) two fluorophores plus at least one Fmoc group allows efficient cellular uptake of the substrates (structures 1,4,6,8,10, 11, and 12).

Other experiments utilizing protease substrates of this invention labeled with two identical fluorophores and at least one additional hydrophobic group such as an Fmoc group fits this paradigm. Replacing this Fmoc group with the less hydrophobic and smaller benzyloxycarbonyl group resulted in lower levels of cellular uptake, but was significantly better than a compound without a hydrophobic group such as DEVD peptide compound structure 5.

These data indicate that Fmoc may be replaced with Benzyloxycarbonyl, Z, or other hydrophobic groups such as Xanthyl (Xan), Trityl (Trt), 4-methyltrityl (Mtt), 4-methoxytrityl (Mmt), 4-methoxy-2,3,6-trimethyl-benzenesulphonyl (Mtr), Mesitylene-2-sulphonyl (Mts), 4,4'-dimethoxybenzhydryl (Mbh),Tosyl (Tos), 2,2,5,7,8-pentamethyl chroman-6-sulphonyl (Pmc), 4-methylbenzyl (MeBzl), 4-methoxybenzyl (MeOBzl), Benzyloxy (BzlO), Benzyl (Bzl), Benzoyl (Bz), 3-nitro-2-pyridinesulphenyl (Npys), 1-(4,4-dimentyl-2,6-diaxocyclohexylidene)ethyl (Dde), 2,6-dichlorobenzyl (2,6-DiCl-Bzl), 2-chlorobenzyloxycarbonyl (2-Cl-Z), 2-bromobenzyloxycarbonyl (2-Br-Z), Benzyloxymethyl (Bom), t-butoxycarbonyl (Boc), cyclohexyloxy (cHxO),t-butoxymethyl (Bum), t-butoxy (tBuO), t-Butyl (tBu), Acetyl (Ac), and Trifluoroacetyl (TFA).

When the acid groups on compound 5, DEVD peptide, were esterified with ethanol, this modified peptide did not show any enhanced cellular uptake by viable cells. Hence the importance of the Fmoc group and the two fluorophor s forming H-type dimers are illustrated by this negative example.

Example 10.

Fluorescence Microscopic Analysis of Cells Incubated with Elastase or Apoptosis-Related Protease Substrates.

The elastase substrate, Fm-K[F1]DAIPNIuSIPK[F1]GY, (where F1 was carboxytetramethylrhodamine, Fm was Fmoc, K[F1] was F1 covalently attached through the epsilon amino group of lysine (K), and Fm-K is the Fmoc group covalently attached at the alpha amino group of the amino terminal lysine residue) was used with HL-60 cells. Cells were incubated with various concentrations of elastase substrate ranging from 10 nM to 10 µM for 5 minutes to 60 minutes. Then the cells were diluted 5-fold with RPMI 1640 medium containing 5% serum or with phosphate buffered saline. The samples were centrifuged and washed once more with 1 ml of washing solution. After centrifugation and removal of the washing solution, cell pellets were loosened with about 25 ul of medium and these cells were transferred to a glass capillary. Capillary tubes were then placed on a glass microscope slide and examined under a fluorescence microscope using standard rhodamine filters.

For apoptosis-related protease activity determination, 10 µM concentration of the compounds listed in Example 8 (compound structures 2 through 13) were incubated with cells for 30 min. to 3 hours. The cells were then washed similarly twice. Using glass capillary tubes, the washed cells were transferred and examined under a fluorescence microscope.

Example 11.

Flow Cytometric Analysis of Cells Incubated with Apoptosis-Related Protease Substrates.

The concentration of substrates used in flow cytometric analysis was 10 µM in RPMI1640 medium containing 4 to 10% fetal calf serum. Cell densities during incubation with the chosen substrates ranged from 50,000 cells per ml to 4,000,000 per ml. Incubation times were from 30 min. to 3 hours at 37 degree C

and incubation volumes were 50 µl to 2 ml. After incubation with substrate for 30 to 60 min, cell suspensions were diluted 10-fold with ice cold Hank's Buffered Saline Solution (HBSS) and then filtered through a nylon fabric sheet. This filtered cell suspension was then subjected to flow cytometric analysis using a 488 nm excitation source. Becton Dickenson, Inc.'s flow cytometer, FacSort, was used in the flow cytometric analysis. Typically, 10,000 to 30,000 events per sample were collected.

Control cells without substrate incubation and the sample with the greatest expected fluorescence signals were used to set the instrument detector parameters. For example after 15 minutes' incubation of Jurkat cells with substrate compound #11 Fm-CGD2D: Fm-K[F1]DBJGDEVDGIDGJPK[F1]GY (where F1 was carboxytetramethylrhodamine; Fm was Fmoc, K[F1] was F1 covalently attached through the epsilon amino group of lysine (K), Nlu was norleucine, B was aminoisobutyric acid, and J was epsilon-aminocaproic acid) an increase of about 10 channels indicating cellular uptake of the substrates was measured. Note substrate #11 was not completely quenched. Hence, a small amount of background fluorescence would be expected from the intact substrate. Signals from the cells that had been activated with 1 ug/ml of ant-Fas antibody, CH11 clone for 1 to 6 hours indicated an increase in peak channel number. As much as a ten-fold increase in fluorescence intensity was observed. When the cells were co-incubated with the CPP32 protease inhibitor ZVAD-fluoromethylketone at 50 µM along with an apoptosis inducing agent, *e.g.*, anti-Fas antibody, this observed increase in fluorescence intensity was eliminated. This indicated that the signal from compound 11 was due to the CPP32 protease activity which was inhibitable by ZVAD-FMK. Hence, the observed fluorescence intensity in each cell as determined by flow cytometric analysis served as a direct measure of the intracellular CPP32 protease activity.

Example 12.

Competitive Substrate Inhibitors Illustrated by Their Effects on Cell Lysate Hydrolysis of Apoptosis-related Protease Substrates.

The level of CPP32 protease activity in the 6 hr ant-Fas-stimulated Jurkat cell lysate was examined using the protease substrate, DEVD-AFC (where AFC is aminofluoromethyl coumarin) 50 µM substrate concentration at 37°C. The

buffer used was 50mM HEPES, pH7.5, 10%w/v sucrose, 0.1%w/v CHAPS.) Fluorescence intensity changes were monitored with an SLM 48000 spectrofluorometer. The hydrolysis rate of DEVD-AFC was found to depend upon the concentration of DEVD, DEVN, and ICE substrates (compounds 5, 7, and 9 in Table 12) present in the reaction mixtures. As the concentrations of DEVD, DEVN, and ICE were raised to 25μM, the rate of DEVD-AFC hydrolysis was decreased. Hence, DEVD, DEVN and ICE substrates do bind to the substrate binding site of target proteases such as CPP32 and act as competitive inhibitors since their hydrolysis rates are slower than that of DEVD-AFC substrate. It was surprising to find that the substrate control peptide with its P_1 residue mutated with a conservative uncharged residue Asn still retained the ability to bind to the protease substrate binding site and exhibit enzyme inhibition.

Example 13.

Substrates Delay and Inhibit Apoptosis Stimuli in Whole Cells.

Jurkat cells are normally grown in 10% fetal calf serum containing RPMI 1640, at 37° C in a 5% CO_2 atmosphere. When the serum content was dropped to 4%, the Jurkat cell growth rate not only slowed down but also a significant number of cells died within 36 hours. The cell density used was about 400,000 cell per ml. After 36 hours, control wells contained about 50% dead cells (trypan blue-positive cells), whereas the wells containing 0.1 or 1.0 μM concentration of compound #11 (Table 12) "Fm-CGD2D" or Fm-K[F1]DBJGDEVDGIDGJPK[F1]GY showed only 10% or 8% nonviable cells. Hence, compound #11 which exhibits efficient cellular uptake slowed down apoptosis in these Jurkat cells where it acted as a CPP32 protease inhibitor or a CPP32 activating protease inhibitor.

Example 14.

Isolation of Intact and Cleaved Substrate Fragments from Cells.

Jurkat cells, which had been induced into apoptosis by the ant-Fas antibody (1 ug/ ml for 2 hours at 37 degree C) were incubated with 10 μM substrate compound #10 Fm-G2D2D. After one hour incubation with this substrate, the cells were washed with 4% serum containing RPMI 1640 medium (1 ml wash solution for every 100 μl of incubation medium). Cells were washed three times, then

solubilized with cell lysis buffer containing Triton X-100. This c ll lysate was then analyzed using a C_4 reverse phase chromatography column and a water/acetonitrile eluent system containing 0.075% trifluoroacetic acid throughout. Analysis showed the presence of intact substrate with two major new peaks that eluted earlier than the intact substrate. The two recovered major peaks showed rhodamine absorption spectra; hence, these correspond to two major substrate fragments that are generated upon protease cleavage of the substrate.

Example 15.

Fluorescence Signal from DEVN Substrates When Mixed with Target Enzyme Containing Solution

DEVN (10 μM), a substrate control peptide, compound 7 of Table 12, was found to be resistant to protease digestion by an apoptosis-activated Jurkat cell lysate. Extensive digestion time did not result in any further increase in fluorescence intensity. HPLC reverse phase analysis of this reaction mixture confirmed the presence of a totally uncleaved substrate. Substitution of the P_1 residue, Asp, by a non-charged amino acid Asn resulted in converting a protease substrate into a protease non-substrate.

This control peptide exhibited competitive substrate inhibition in the experiment as described in Example 12. In addition, fluorescence intensity monitoring as a function of time after addition of cell lysate showed a significant increase in fluorescence intensity initially but after 15 minutes this initial intensity level stabilized. Recalling that there was no substrate cleavage by the proteases present in the cell lysate, the best explanation of this initial fluorescence intensity is due to the DEVN substrate binding to the protease and the substrate undergoing a conformational change. This conformational change involving the substrate's backbone also affects the conformation of two covalently attached fluorescent dye molecules with respect to each other in terms of mean distance and relative orientation. The degree of fluorescence quenching of these two fluorophores in the substrate structure has been found to be sensitive to their distance and the specific orientation with respect to their dipoles. Hence, any conformational change that affects these two aspects of the fluorescence reporting molecules would be expected to affect the fluorescence quenching as well. Thus, conformational changes induced by a substrate binding to a protease's substrate binding site is

reflected in the observed initial fluorescence intensity changes, *i.e.*, an increase in its fluorescence intensity. Since the substrate cannot be cleaved, the initial fluorescence intensity increase levels off. One can utilize this observed fluorescence intensity increase due to conformational change of the substrate rather than substrate cleavage as a new kind of readout such as degree of association between the substrate and its target binding molecule.

Example 16.

Variation of Hydrolysis Rates Induced by Varying the Flexibility of the Protease Recognition Domain by Various Conformation Determining Domain (CDR) Amino Acid Sequences.

Protease cleavage sites of physiologically relevant substrates for any give protease may be classified into two cases. One is the serine protease inhibitors such as neutrophil elastase inhibitor or alpha 1 antitrypsin where the elastase recognition sequence is rather rigidly held by the remaining inhibitor molecule. Upon cleavage by elastase, this protease reactive site and its newly formed terminal residues undergoes significant conformational change as evidenced by high resolution crystallographic structural analyses of reactive and cleaved inhibitor structures. In the second class of protease cleavage sites the cleavage sequences are in domains where conformations are not well defined or there is a significant amount of flexibility, as much as in free linear peptides. It is said that some degree of defined conformation or a less than maximally available backbone flexibility difference between two potential substrates results in a given protease's preference for one substrate over another.

Compounds 4 (Fm-DEVD), 10 (Fm-G2D2D), and 11 (Fm-CGD2D) illustrate how one can introduce varying amount of constrained conformational space or flexibility into a given substrate with the same protease recognition domain but with different conformation determining domains or regions (CDRs) while retaining the bent forming function of the CDRs. This example illustrates how one can vary relative rigidity or flexibility of the central protease recognition domain by changing the CDR's conformational flexibility or rigidity.

The parent compound Fm-DEVD has the following composition: Fmoc-K[F1]DB**DEVDGID**PK[F1]GY. The bold face underlined letters are the protease recognition sequence consisting of 7 amino acid residues. Compound #10

contains two glycine extensions at both ends of this protease recognition sequence. The central protease recognition domain now is 8 residue long GDEVDGID, since the glycine residue at the amino terminus is a part of native sequence. The two glycine residues which are inherently more flexible than other amino acids, *e.g.*, alanine, provide less conformational constraint or, conversely, more flexibility than compound 4 (Table 12) and thereby permit greater flexion when combined with Aib or Pro residues. Additional insertion of amino caproic acid at both termini with five methylene groups in addition to the one present in glycine provides further relaxation of the constrained conformation and, thus, greater flexibility for the protease recognition domain, GDEVDGID. This progression of flexibility resulted in an increased hydrolysis rate with the CPP32 protease since CPP32 recognizes a more flexible protease recognition domain than does elastase. Support for this statement is that the CPP32 protease cleavage site in the proform of its physiological substrate, poly(ADP-ribose) polymerase, PARP, is located between two well-folded domains. Hence, it is expected that such a protease cleavage site would not be rigidly held or its conformation would be expected to be less defined than the remaining molecule. Hence, in order to provide these structural features to the substrate, introduction of flexible residues such as glycine, epsilon amino caproic acid, beta alanine, and amino butyric acid would be expected to play important roles in regulating the backbone flexibility of the substrate's central protease recognition domain. These additional preferred residues for the conformation determining domain are also expected to provide the needed bend-inducing influence.

The observed varied hydrolysis rates for these three substrates point to success in regulating the flexibilities of the protease recognition domains. This, is reflected in the observed difference in proteolysis rates while maintaining the appropriate orientation for the two fluorophores to interact with each other through space. The importance of this conformation determining domain in providing a means to regulate flexibility as well as allowing the amino and carboxyl termini to orient in space appropriately close is exemplified by these compounds (4, 10, and 11).

Tnese examples provide a tetrapeptide and a pentapeptide comprising Lys-Asp-Aib-Gly or Lys-Asp-Aib-Ahx-Gly where Ahx is episilon amino caproic acid (*i.e.* NH_2-$(CH_2)_5$-COOH). The fluorophore is attached to episilon amino

group of the lysine residue. The carboxyl terminal CDR domain is defined as a tripeptide Gly-Pro-Lys and a tetrapeptide Gly-Ahx-Pro-Lys. The hydrolysis rate was increased by 3-fold between compounds 4 (Fm-DEVD: Fm-K[F1]DBDEVDGIDPK[F1]GY) and 10 (Fm-G2D2D: Fm-K[F1]DBGDEVDGIDGPK[F1]GY])

As illustrated in Figure 5, the hydrolysis rate was further increased by *ca.* 3-fold over the above glycine residue insertion with the amino caproic amino acid (Ahx) addition, compound 11 (Fm-CGD2D: Fm-K[F1]DB Ahx GDEVDGIDG Ahx PK[F1]GY). Hence, overall at least a 9-fold increase in substrate hydrolysis rate was accomplished (compounds 4 and 11, Table 12).

Example 18

Structural Characteristics of Fluorophores Which Form Intramolecular H-type Dimers in a Protease Substrate.

The strongest correlations between H-dimer formation and structural elements of various potential fluorophores for use in the homo-doubly labeled fluorogenic compositions of this invention are in order: delocalized charge, symmetry, and transition dipole magnitude. Hydrophobicity was not observed to be a major determinant in this type of dimerization.

In the experiments described herein, a new class of profluorescent protease substrate was designed and synthesized. These new fluorogenic indicators have spectral properties that fit the exciton model; More specifically, spectra of these polypeptides which were doubly labeled with rhodamines showed a blue-shifted absorption peak and fluorescence quenching, both indicators of H-dimer formation.

For example, NoreFes, an undecapeptide which is cleaved by the serine protease elastase, was homodoubly labeled on opposite sides of its cleavage site with six fluorophores in order to identify structural elements of dyes which influence intramolecular H-type dimer formation. Absorption and fluorescence spectra of these six substrate obtained before and after enzymatic cleavage suggest that the presence of a delocalized charge followed by symmetry and then magnitude of the lowest energy electronic transition dipole are important factors in dimer formation. Surprisingly, there was no evidence that hydrophobic interactions were important in the fluorophores used in this study.

The six fluorophores used in this study were rhodamine-X, tetramethylrhodamine, fluorescein, diethylaminocoumarin, hydroxycoumarin and pyrene.

While the xanthene components of these two rhodamines (rhodamine-X, tetramethylrhodamine) have the same charge and symmetric structure, the distinguishing characteristics between them are a higher transition dipole magnitude and lower hydrophobicity of the tetramethylrhodamine. One notes that the spectrum of the intact tetramethylrhodamine-derivatized substrate shows a more prominent change than that of rhodamine-X when comparing the absorption spectra of the two doubly-labeled intact peptides with those from the respective cleaved solution.

As noted above, in contrast to the two rhodamine derivatives where a charge of +1 is localized over each of the xanthene structure, the three conjugated ring component of the fluorescein was uncharged at pH 9. The lack of any significant shape changes in the absorption spectra after separation of the dyes (fluorescein) by cleavage of the peptide suggests a role for charge in H-dimer formation. The less pronounced, but nevertheless finite quenching observed with this derivative points toward a diminished but finite degree of interaction between two fluorescein compared with interactions between either of the two rhodamines is consistent with previous studies of xanthene in solution where the association constant for dimer formation for fluorescein is four order of magnitude lower than that for rhodamines.

The influence of dye symmetry was next examined using two coumarins, *i.e.* diethylaminocoumarin and hydroxycoumarin. This class of molecules contains no symmetrical elements. The diethylaminocoumarin bears a positive charge delocalized over its two conjugated rings, similar to the rhodamines and the hydroxycoumarine is neutral at pH 9, similar to fluorescein. The spectrum of diethylaminocoumarin-labeled NorFes exhibits a blue shift of 11 nm while that of hydroxycoumarin-labeled NorFes shows just a slight blue shoulder. The respective degree of quenching, 76% and 28% of the intact peptides relative to the cleaved solutions is consistent with the importance of delocalized charge. Comparing the less pronounced spectral changes of the diethylaminocoumarin-derivatized peptide with those of the xanthene gives support to the role of symmetry as an important element in H-dimer formation.

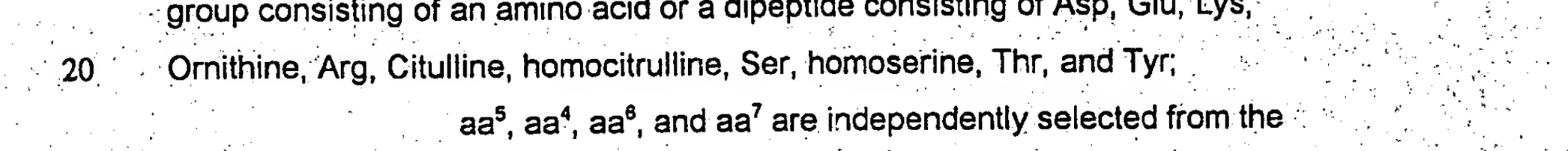
Finally, the role of hydrophobicity was studied using pyrene, a fluorophore with S2 symmetry containing only carbons and hydrogens. No spectral changes were observed in either the absorption or the fluorescence mode and the magnitude of the transition dipole is extremely small. These results provide evidence against a dominant role for hydrophobicity in H-dimer formation.

In summary, the strongest correlations between H-dimer formation and structural elements are in order: delocalized charge, symmetry, and transition dipole magnitude. Hydrophobicity was not observed to be a major determinant in this type of dimerization.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

WHAT IS CLAIMED IS:

1. a fluorogenic composition for the detection of the activity of a protease, said composition having the formula:



wherein, P is a peptide comprising a protease binding site for said protease, said binding site consisting of about 2 to about 8 amino acids;

F^1 and F^2 are fluorophores and F1 is attached to the amino terminal amino acid and F2 is attached to the carboxyl terminal amino acid;

S^1 and S^2, when present, are peptide spacers ranging in length from 1 to about 50 amino acids and S^1, when present, is attached to the amino terminal amino acid and S^2, when present, is attached to the carboxyl terminal amino acid;

i, j, k, l, m, n, o, p, q, and r are independently 0 or 1;

aa^1 and aa^{10} are independently selected from the group consisting of lysine, ornithine and cysteine;

aa^2, aa^3, aa^8, and aa^9 are independently selected from the group consisting of an amino acid or a dipeptide consisting of Asp, Glu, Lys, Ornithine, Arg, Citulline, homocitrulline, Ser, homoserine, Thr, and Tyr;

aa^5, aa^4, aa^6, and aa^7 are independently selected from the group consisting of proline, 3,4-dehydroproline, hydroxyproline, alpha aminoisobutyric acid and N-methyl alanine;

X is selected from the group consisting of Gly, βAla, γAbu ,Gly-Gly, Ahx, βAla- Gly, βAla-βAla, γAbu-Gly, βAla-γAbu, Gly-Gly-Gly, γAbu-γAbu, Ahx-Gly, βAla-Gly-Gly, Ahx-βAla, βAla-βAla-Gly, Gly-Gly-Gly-Gly, Ahx-γAbu, βAla-βAla, γAbu-βAla-Gly, γAbu-γAbu-Gly, Ahx-Ahx, γAbu-γAbu-βAla, and Ahx-Ahx-Gly;

Y is selected from the group consisting of Gly, βAla, γAbu, Gly-Gly, Ahx, Gly-βAla, βAla-βAla, Gly-γAbu, γAbu-βAla, Gly-Gly-Gly, γAbu-γAbu, Gly-Ahx, Gly-Gly-βAla,βAla-Ahx, Gly-βAla-βAla, Gly-Gly-Gly-Gly, γAbu-Ahx, βAla-βAla-βAla, Gly-βAla-γAbu, Gly-γAbu-γAbu, Ahx-Ahx, βAla-γAbu-γAbu, and Gly-Ahx-Ahx;

when i is 1, S^1 is joined to aa^1 by a peptide bond through a terminal alpha amino group of aa^1; and

when r is 1, S^2 is joined to aa^{10} by a peptide bond through a terminal alpha carboxyl group of aa^{10}.

2. The composition of claim 1, wherein P is selected from the group consisting of: TGRTG, DEVDGID, DEVNGID, EVDGID, ADGID, DEVDGID, AIPMSI, GDEVDGID, GDEVDGIN, ADGID, GNEVDGID, GNEVDGIN, ODEVDGID, dODEVDGID, WDEVDGID, dWDEVDGID, dOdODEVDGID, dWdWDEVDGID, YVADGID, YVADGIN, YVANGIN, YVADGID, YVANGIN, and dYVADGIN.

3. The composition of claim 1, wherein P is selected from the group consisting of: LVEIDNG, LVEINNG, GIETDSGVDD, GIETNSGVDD, GIETNSGV, GIETDSGV, GSESMDSGISLD, GSESMDSG, DVVCCSMS, DVVCDSMS, DVVCSdMS, DVVCCPdMS, EDVVCCS, EDdVVCCP, EDdVVCDP, DdVVCCSdMS, DVdVCDSdMS, DdVVCCPdMS, DVVCCSM, DVVCDSM, VCCSM, and VCDSM.

4. The composition of claim 1, wherein P is selected from the group consisting of: DEMEECSQHL, DEMEECPQHL, EMEECSQHL, EMEECPQHL, EMEEDSQHL, VMTGRTG, VdMTGRTG, VMTGRG, VdMTGRG, VMTGRVG, VdMTGRVG, VMTGRAG, VdMTGRAG, SEVNLDAEF, SEVKLDAEF, SEVKMDAEF, SEVKMDDEF, SEVNLDDEF, GVVIATVIVIT, YGVVIATVIVIT, VIATVI, YGVVIA, QQLLNH, SIQYTY, SSQYSN, and SSIYSQ.

5. The composition of claim 1, wherein F^1 and F^2 are the same fluorophore.

6. The composition of claim 5, wherein said F^1 and F^2 have an excitation wavelength between about 315 nm and about 700 nm.

7. The composition of claim 1, wherein the F^1 molecule is attached through either an α-amino group of the aa^1 amino acid or through a side chain amino group of the aa^1 amino acid, or through a sulfhydryl group of a side chain of the aa^1 amino acid.

8. The composition of claim 1, wherein the F^2 molecule is attached either through a side chain amino group of the aa^{10} amino acid, through a carboxyl

group of the aa^{10} amino acid, or through a sulfhydryl group of a side chain of the aa^{10} amino acid.

9. The composition of claim 1, wherein said fluorophore is selected from the group consisting of carboxytetramethylrhodamine, carboxyrhodamine-X and diethylaminocoumarin, 9-(2,5-dicarboxyphenyl)-3,6-bis-(dimethylamino)xanthylium chloride (5-TMR), 9-(2,6-dicarboxyphenyl)-3,6-bis-(dimethylamino)xanthylium chloride (6-TMR), 9-(2-carboxyphenyl)-2,7-dimethyl-3,6-bis(ethylamino)xanthylium, 9-(2-carboxyphenyl)-3,6-bis(dimethylamino)xanthylium, and 9-(2-carboxyphenyl)-xanthylium..

10. The composition of claim 1, wherein said composition bears a hydrophobic group.

11. The composition of claim 10, wherein said hydrophobic group is selected from the group consisting of: Fmoc, benzyloxycarbonyl, Xanthyl (Xan), Trityl (Trt), 4-methyltrityl (Mtt), 4-methoxytrityl (Mmt), 4-methoxy-2,3,6-trimethyl-benzenesulphonyl (Mtr), Mesitylene-2-sulphonyl (Mts), 4,4'-dimethoxybenzhydryl (Mbh),Tosyl (Tos), 2,2,5,7,8-pentamethyl chroman-6-sulphonyl (Pmc), 4-methylbenzyl (MeBzl), 4-methoxybenzyl (MeOBzl), Benzyloxy (BzlO), Benzyl (Bzl), Benzoyl (Bz), 3-nitro-2-pyridinesulphenyl (Npys), 1-(4,4-dimentyl-2,6-diaxocyclohexylidene)ethyl (Dde), 2,6-dichlorobenzyl (2,6-DiCl-Bzl), 2-chlorobenzyloxycarbonyl (2-Cl-Z), 2-bromobenzyloxycarbonyl (2-Br-Z), Benzyloxymethyl (Bom), t-butoxycarbonyl (Boc), cyclohexyloxy (cHxO),t-butoxymethyl (Bum), t-butoxy (tBuO), t-Butyl (tBu), Acetyl (Ac), and Trifluoroacetyl (TFA).

12. The composition of claim 11, wherein said hydrophobic group is Fmoc.

13. The composition of claim 11, wherein said hydrophobic group is attached to the amino terminus of the molecule.

14. The composition of claim 1, wherein said composition is selected from the group consisting of compositions named PAI-2, DEVD, DEVN, PARP, ICE,

Fm-DEVD, Fm-FmDEVN, Fm-PARP, Fm-KNFES, Fm-G2D2D, Fm-CGD2D, Z-CGD2D, and Fm-ICE.

15. a method of detecting the activity of a protease, said method comprising contacting said protease with a composition of claim 1.

16. The method of claim 15, wherein said contacting is in a histological section.

17. The method of claim 15, wherein said contacting is in a cell suspension derived from a biological sample selected from the group consisting of a tissue, blood, urine, saliva, lymph, biopsy.

18. The method of claim 15, wherein said detecting is by a method selected from the group consisting of fluorescence microscopy, fluorescence microplate reader, flow cytometry, fluorometry, absorption spectroscopy.

19. a method of detecting a change in conformation of a molecule, said method comprising

providing a first molecule having attached thereto a first fluorophore and a second fluorophore wherein said first fluorophore and said second fluorophore are the same species of fluorophore, and said fluorophores are juxtaposed at a distance sufficient for the interaction of said fluorophores to detectably reduce the fluorescence intensity of each of said fluorophores as compared to the fluorescence intensity of a single fluorophore attached to the molecule at the same location; and

detecting the change in fluorescence as the spacing between said fluorophores is increased by said change in conformation of said molecule.

20. The method of claim 19, wherein said fluorophores form an H-type dimer before the increase in spacing.

21. The method of claim 19, wherein said fluorophores are at a distance of less than approximately 10 Å from each other.

22. The method of claim 19, wherein said fluorophores are selected from the group consisting of carboxytetramethylrhodamine, carboxyrhodamine-X and diethylaminocoumarin, tetramethylrhodamine, diethylrhodamine, and rhodamine 110.

23. The method of claim 19, wherein said change in conformation is cleavage of said molecule into two different molecules each bearing one of the fluorophores.

24. The method of claim 19, wherein said change in conformation is produced by binding of a second molecule to said first molecule.

25. The method of claim 19, wherein said molecule is selected from the group consisting of a nucleic acid, a polysaccharide, a peptide, a protein, a lipid, a phospholipid, a glycolipid, a glycoprotein, a steroid.

26. The method of claim 19, wherein said molecule is a nucleic acid and said change in conformation is produced by hybridization of said nucleic acid to another nucleic acid.

27. The method of claim 19, wherein said molecule is a nucleic acid and said change in conformation is produced by cleavage of said nucleic acid.

28. The method of claim 19, wherein said molecule is a polysaccharide and said change in conformation is produced by cleavage of said polysaccharide.

29. a fluorogenic composition for the detection of a change in conformation of said composition, said fluorogenic composition comprising a molecule having attached thereto a first fluorophore and a second fluorophore wherein said first fluorophore and said second fluorophore are the same species of fluorophore, and said fluorophores are juxtaposed at a distance sufficient for the interaction of said fluorophores to detectably reduce the fluorescence intensity of each of said fluorophores as compared to the fluorescence intensity of each individual fluorophore attached to the molecule at the sam location.

30. A method of delivering a molecule into a cell, said method comprising:

providing said molecule attached to a hydrophobic group and to either two fluorophores or to at least one fused ring structure; and

contacting said cell with said molecule whereby said molecule enters said cell.

31. The method of claim 30, wherein said molecule is selected from the group consisting of a nucleic acid and a polypeptide.

32. The method of claim 30, wherein said fused ring structure is a biologically inactive steroid.

33. The method of claim 30, wherein said hydrophobic group is selected from the group consisting of: Fmoc, benzyloxycarbonyl, Xanthyl (Xan), Trityl (Trt), 4-methyltrityl (Mtt), 4-methoxytrityl (Mmt), 4-methoxy-2,3,6-trimethyl-benzenesulphonyl (Mtr), Mesitylene-2-sulphonyl (Mts), 4,4'-dimethoxybenzhydryl (Mbh),Tosyl (Tos), 2,2,5,7,8-pentamethyl chroman-6-sulphonyl (Pmc), 4-methylbenzyl (MeBzl), 4-methoxybenzyl (MeOBzl), Benzyloxy (BzlO), Benzyl (Bzl), Benzoyl (Bz), 3-nitro-2-pyridinesulphenyl (Npys), 1-(4,4-dimentyl-2,6-diaxocyclohexylidene)ethyl (Dde), 2,6-dichlorobenzyl (2,6-DiCl-Bzl), 2-chlorobenzyloxycarbonyl (2-Cl-Z), 2-bromobenzyloxycarbonyl (2-Br-Z), Benzyloxymethyl (Bom), t-butoxycarbonyl (Boc), cyclohexyloxy (cHxO),t-butoxymethyl (Bum), t-butoxy (tBuO), t-Butyl (tBu), Acetyl (Ac), and Trifluoroacetyl (TFA).

34. The method of claim 30 wherein, said fluorophores are selected from the group consisting of: of carboxytetramethylrhodamine, carboxyrhodamine-X and diethylaminocoumarin.

35. The method of claim 30 wherein, said cell is a mammalian cell.

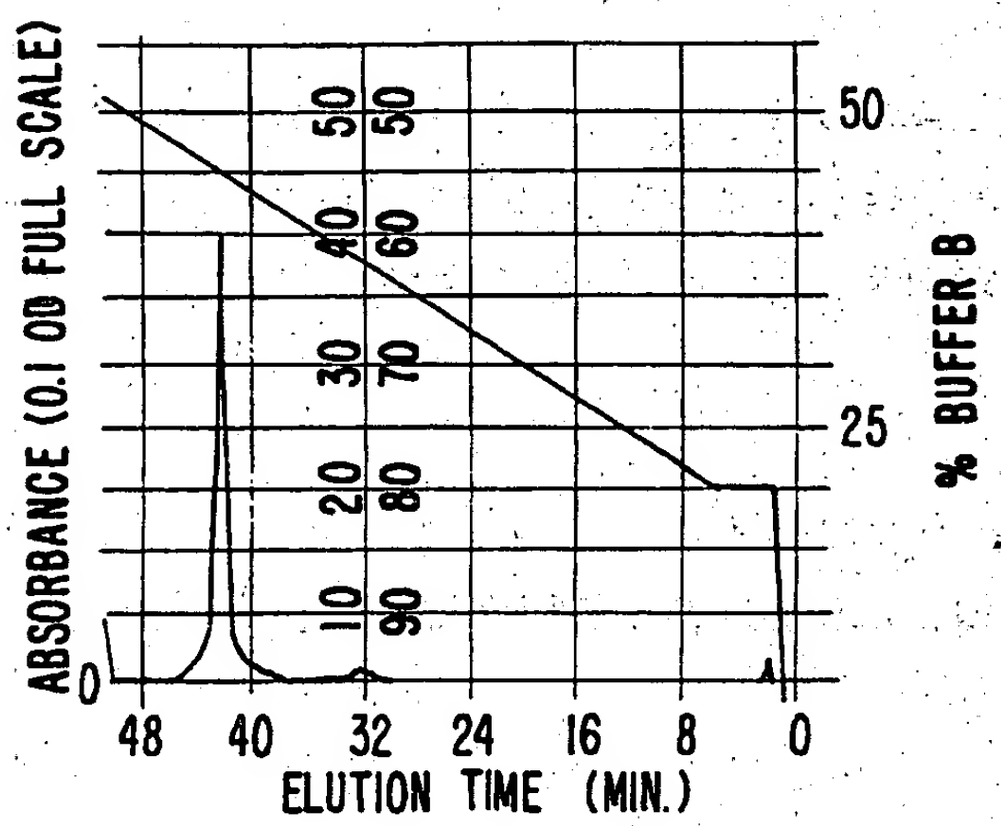


FIG. 1A.

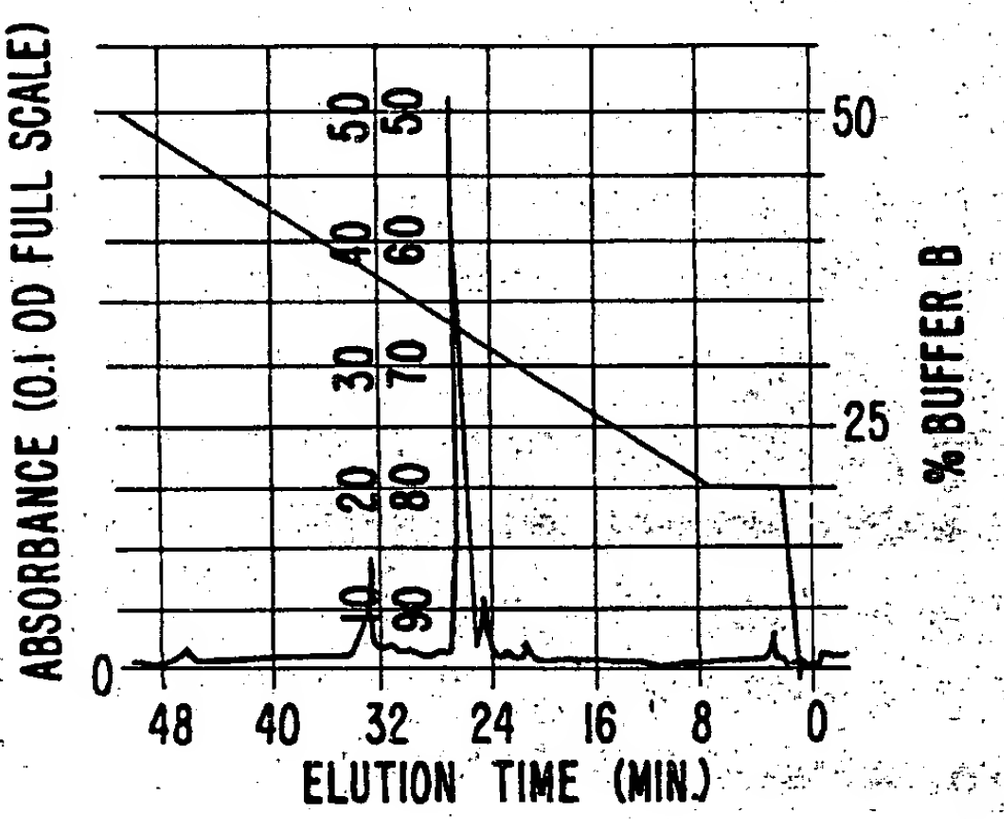


FIG. 1B.

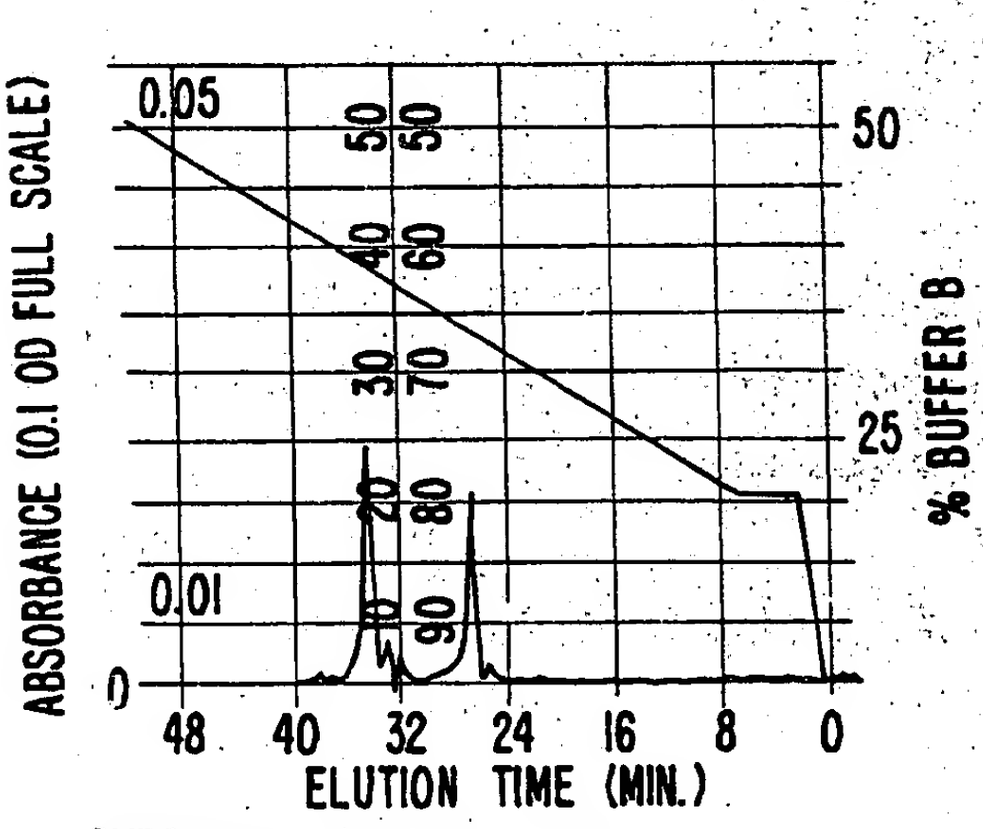


FIG. 1C.

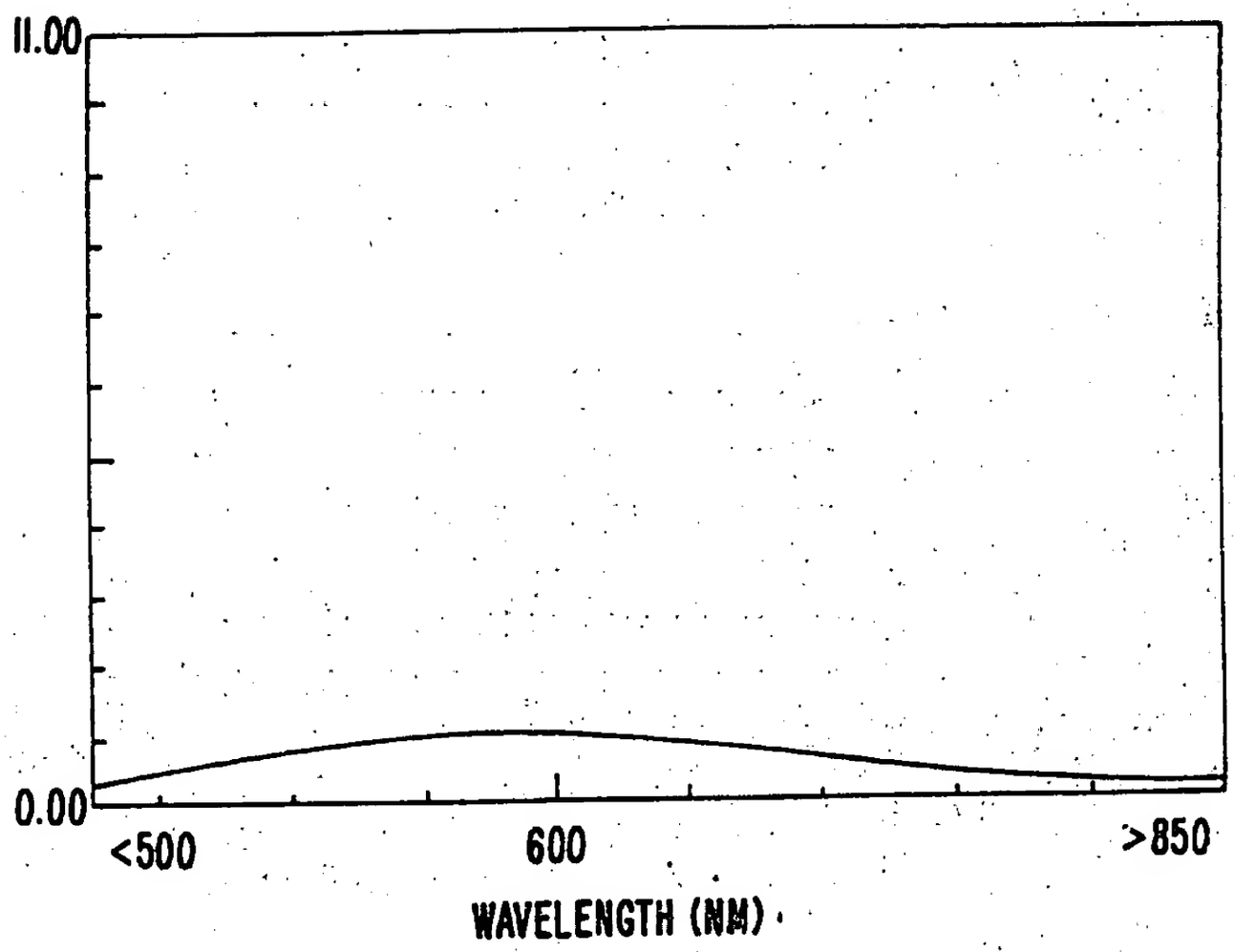

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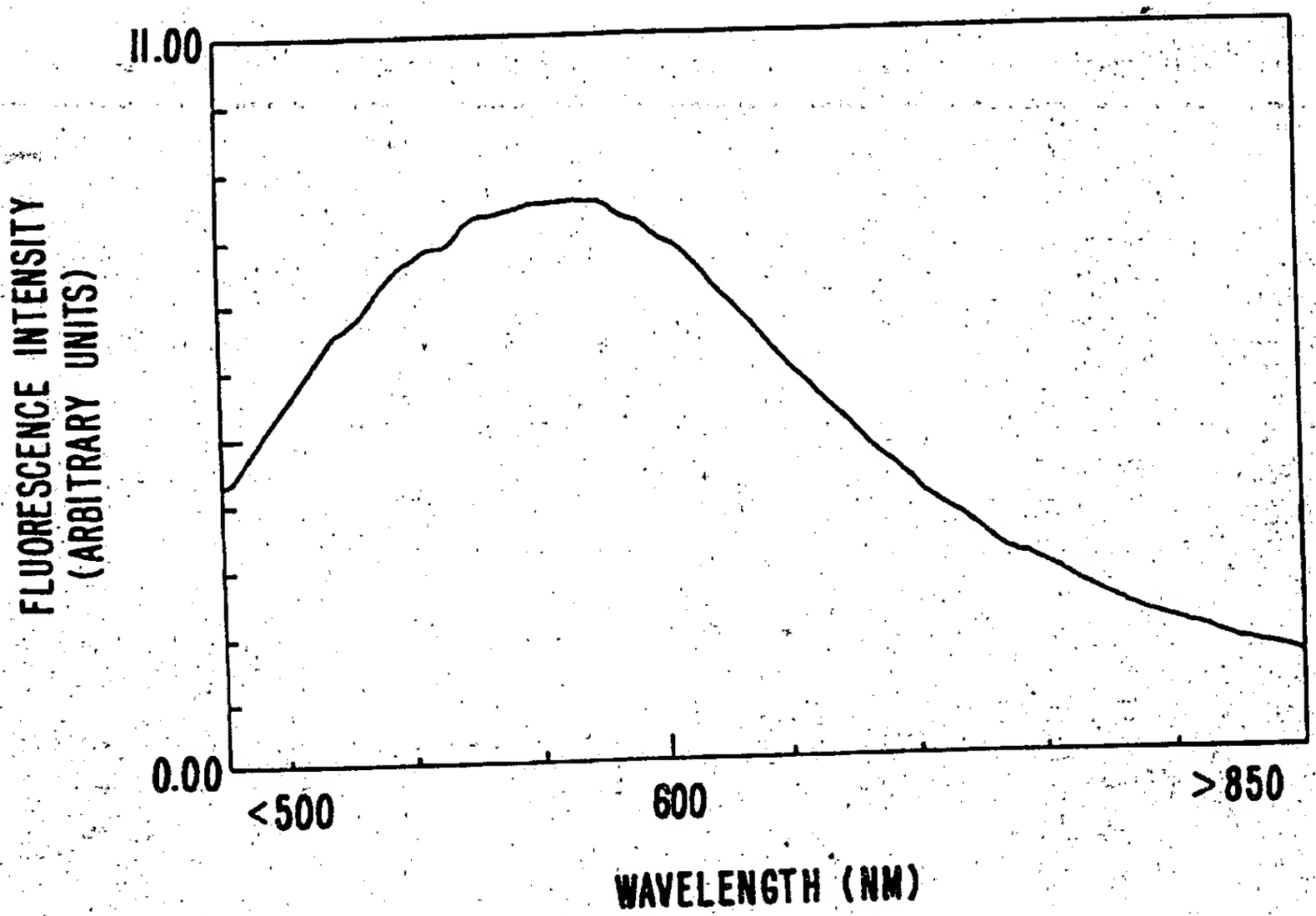
FIG. 2A.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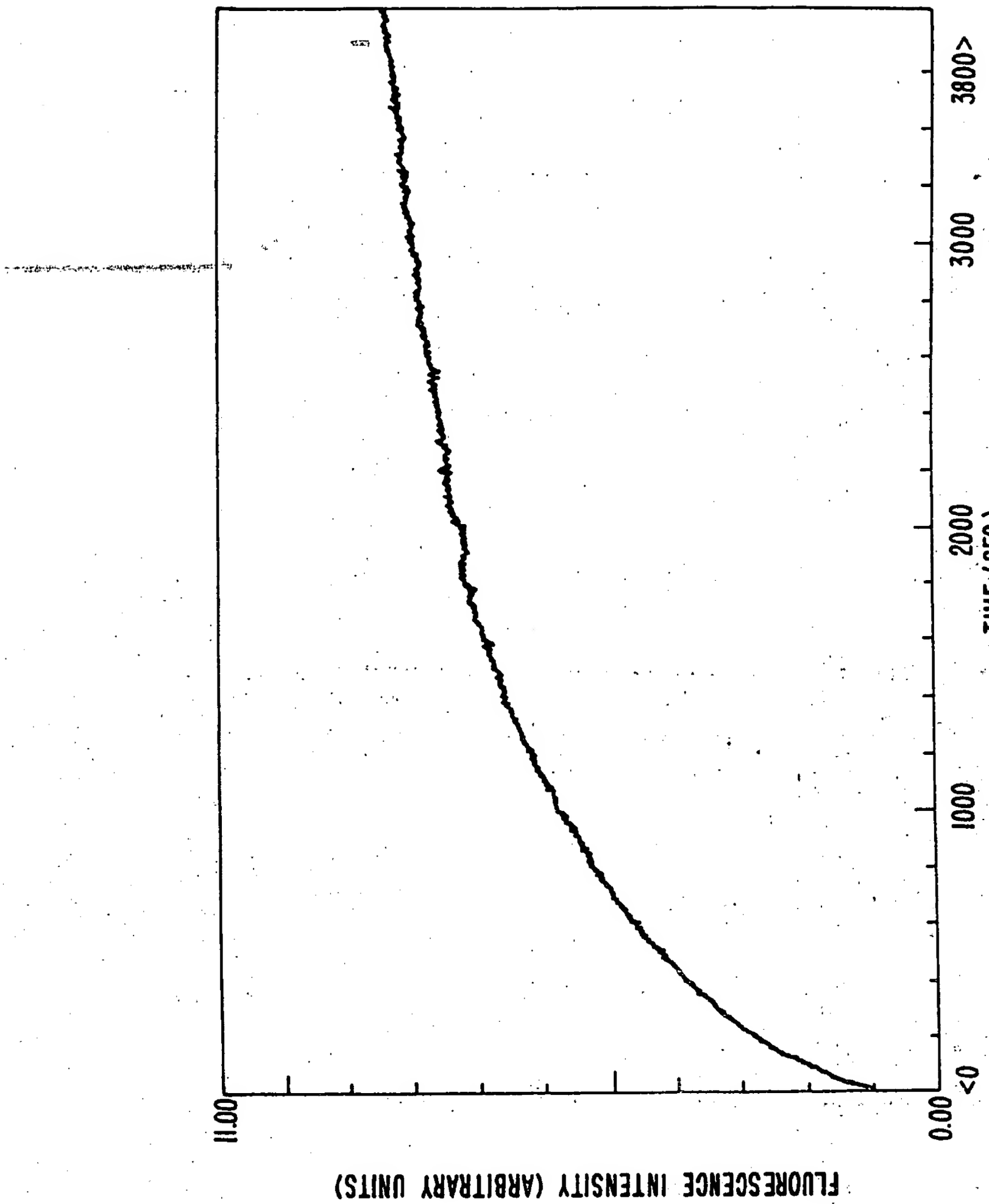
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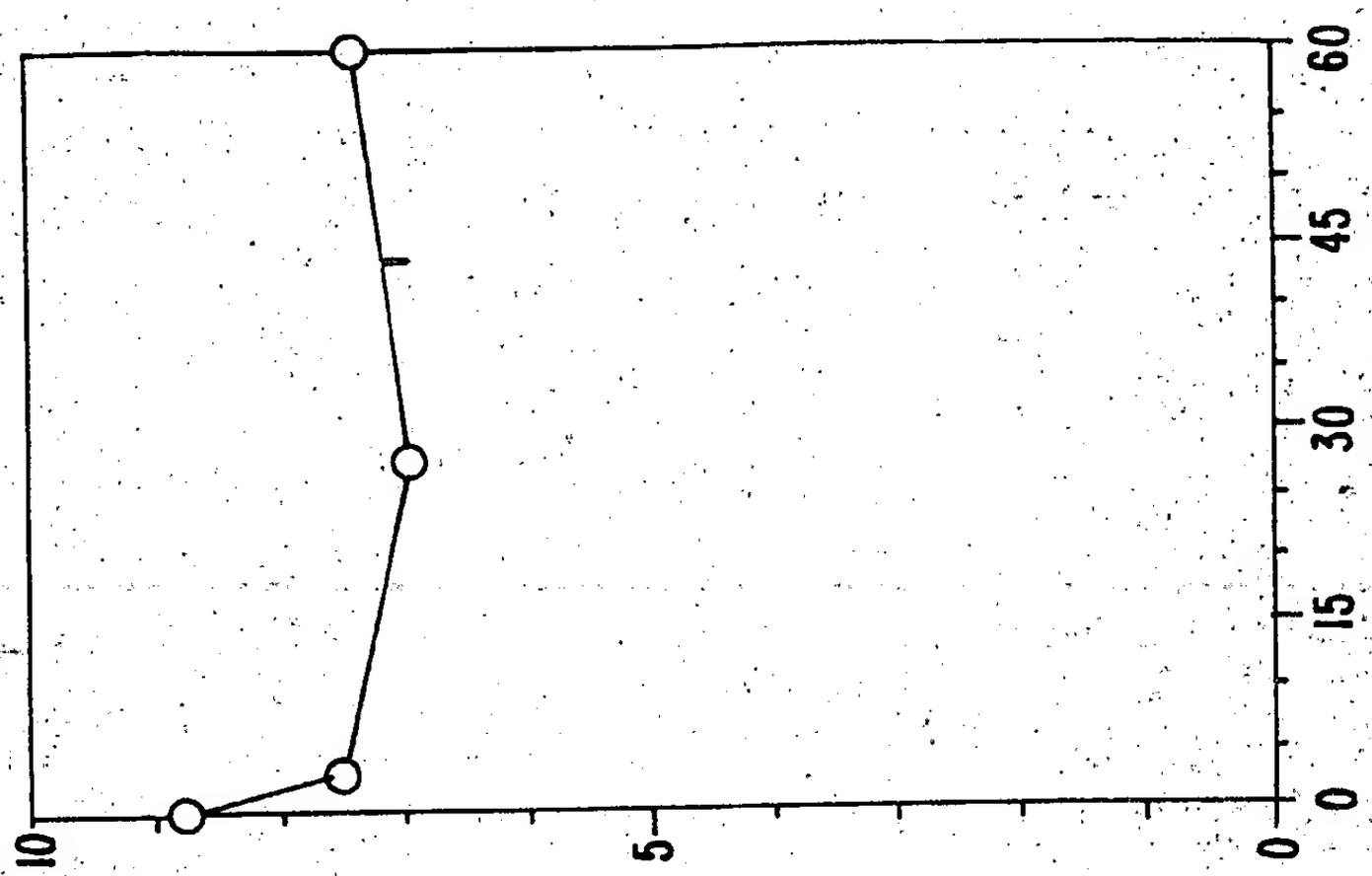
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FIG. 4B.

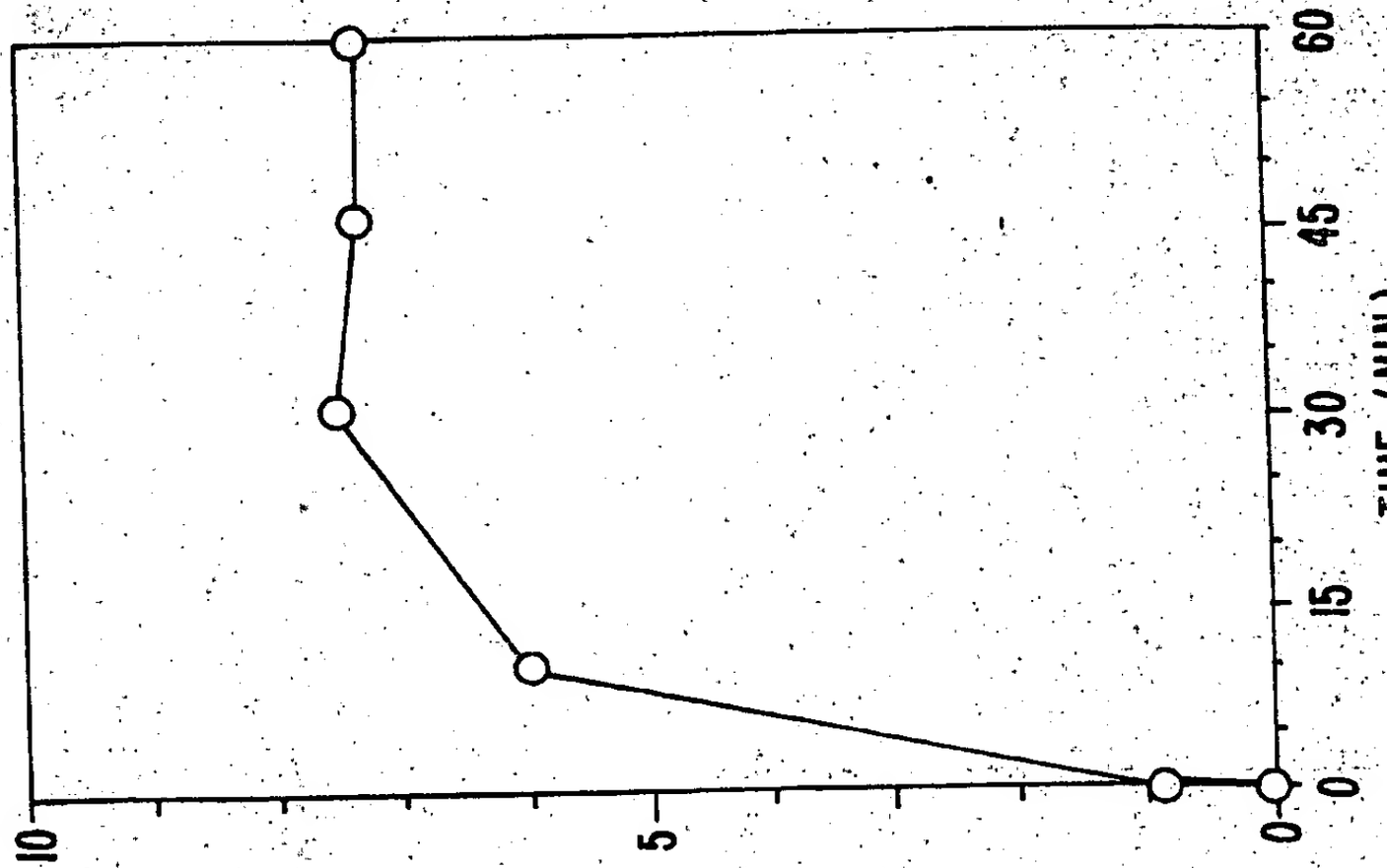
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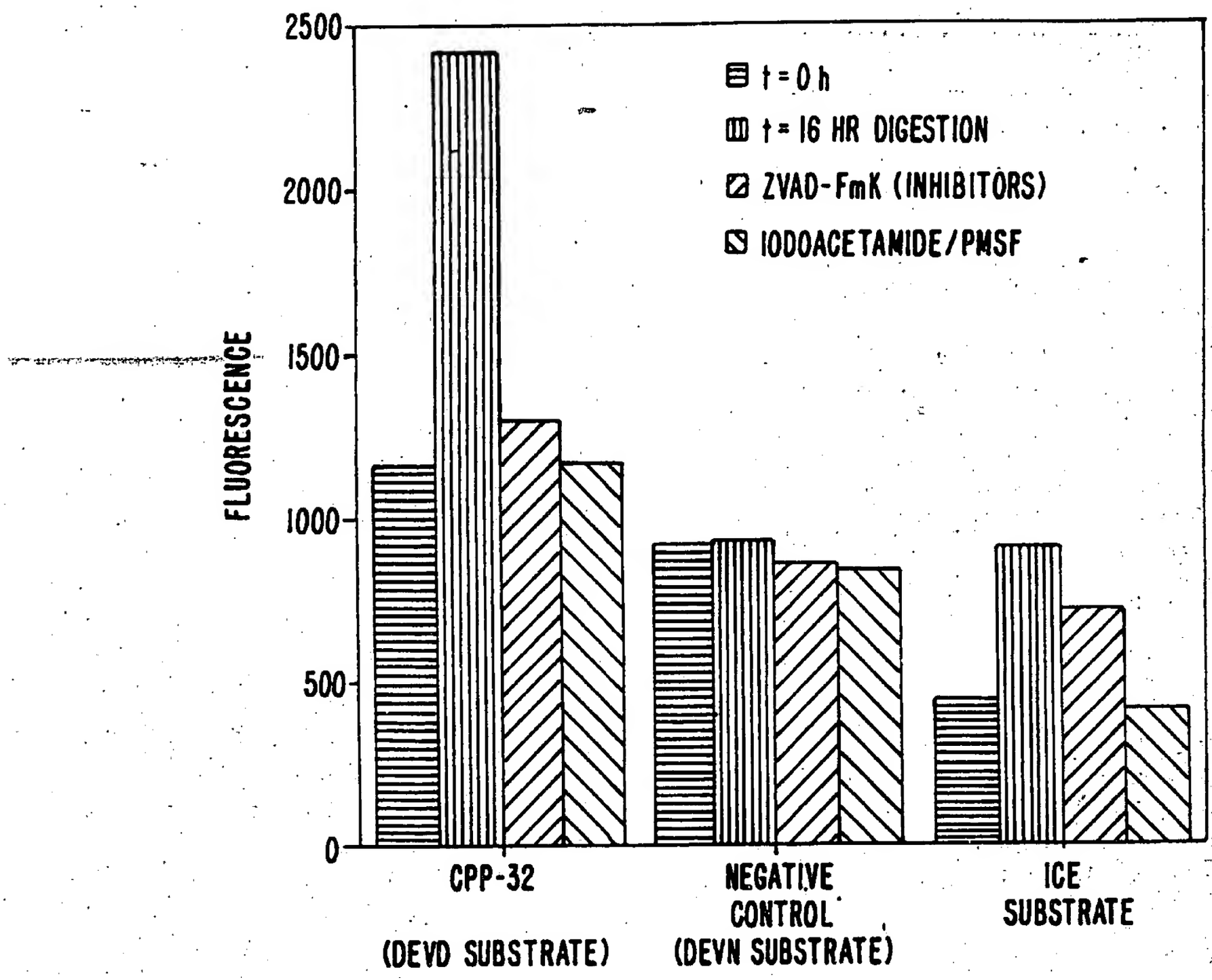
FIG. 4A.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(DEVD SUBSTRATE)
NEGATIVE
CONTROL
(DEVN SUBSTRATE)
ICE
SUBSTRATE

FIG. 5.

INTERNATI NAL SEARCH REPORT

International application No.
PCT/US98/03000

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/00, 1/37; G10N 33/00
US CL :435/23, 7.2, 7.5, 7.6; 436/172; 530/300
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/23, 7.2, 7.5, 7.6; 436/172; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN
search terms: protease, fluorophore, proximity, assay

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 5,605,809 A (KOMORIYA et al.) 25 February 1997, see entire document.	1-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
09 APRIL 1998

Date of mailing of the international search report
02 JUN 1998

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Form PCT/ISA/210 (second sheet)(July 1992)*